MECHANISM OF BLOCK AND BEHAVIORAL EFFECTS OF THE *N*-METHYL-D-ASPARTATE RECEPTOR ANTAGONISTS MEMANTINE AND KETAMINE

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Pharmacological inhibition of NMDA receptor activity by ketamine is accompanied by pyschotomimetic side-effects; however, the Alzheimer's disease therapeutic memantine blocks NMDA receptor activity without debilitating side-effects. This dissertation provides electrophysiological and behavioral characterizations of these two NMDA receptor antagonists in an attempt to understand the unique therapeutic utility of memantine. The following work explores memantine and ketamine inhibition at NMDA receptors, their main site of action, with a focus on the mechanism of inhibition and receptor subtype selectivity in physiologically relevant conditions. This research shows NMDA receptors possess a second binding site at which memantine, but not ketamine, can inhibit activity. The research also shows the dramatic effect physiological concentrations of magnesium has on the ability of these drugs to inhibit NMDA receptor activity. Behavioral and cognitive effects of memantine and ketamine are also assessed and compared directly in rat. The effects of memantine and ketamine in rat were found to be similar at the low doses tested and more divergent as dose increased. Furthermore, memantine's effects appeared to be more pronounced and longer-lasting than those of ketamine. These findings demonstrate the importance of considering the physiological environment in which a drug acts, as well as the principles of drug action, when examining the effects of a drug on central nervous system activity.

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1.0 BACKGROUND AND INTRODUCTION

1.1 GENERAL INTRODUCTION

Most excitatory synaptic communication occurring within the vertebrate central nervous system is mediated by the glutamate receptor family of ligand-gated ion channels. *N*-methyl-D-aspartate (NMDA) receptors are a subtype of glutamate receptors that possess unique properties that allow them to contribute to a variety of physiological (development, learning and memory) and pathological (ischemic cell death, many neurodegenerative disorders) processes (Bliss and Collingridge, 1993; Dingledine et al., 1999; Olney, 2003). Alteration of the ability of NMDA receptors to transmit information reliably can result in behavioral and cognitive disturbances (Lodge et al., 2002).

Pharmacological modulation of NMDA receptor-mediated neurotransmission has generally been accompanied by debilitating psychotomimetic side-effects (Palmer, 2001; Lipton, 2004b). While not widely used clinically, some NMDA receptor antagonists (such as ketamine and phencyclidine (PCP) have been crucial in furthering our understanding of the underlying pathologies of schizophrenia. Ketamine, as well as PCP, reliably reproduces positive (hallucinations and delusions), negative (decreased affect), and disorganizational (thought disorder) symptoms of schizophrenia in healthy human adults as well as worsening symptoms in already afflicted individuals (Krystal et al., 1999b). Memantine, another NMDA receptor

antagonist, with a mechanism of inhibition comparable to that of ketamine, lacks psychotomimetic side-effects and also has shown therapeutic utility by decreasing the cognitive decline associated with late stages of moderate-to-severe Alzheimer's disease. The goal of this work is to evaluate the mechanism of action and behavioral effects of NMDA receptor inhibition by memantine and ketamine to improve our understanding of the therapeutic potential NMDA receptor antagonism may hold.

1.2 NMDA RECEPTOR PROPERTIES

1.2.1 Gene products and expression patterns

NMDA receptor subunit proteins are produced from seven different genes that encode three subunit families: NR1, NR2, and NR3 (Moriyoshi et al., 1991; Monyer et al., 1992; Ishii et al., 1993; Monyer et al., 1994; Ciabarra et al., 1995; Sucher et al., 1995; Dingledine et al., 1999; Chatterton et al., 2002; Matsuda et al., 2003). One gene (*GRIN1*) encodes the NR1 subunit, with eight splice variants produced by alternative splicing (Zukin and Bennett, 1995). Four genes (*GRIN2A-D*) produce distinct NR2 subunits, NR2A-D. Functional NMDA receptors require the presence of NR1 and at least one of the four NR2 subtypes. NR3 subunits are the products of two genes (*GRIN3A-B*) and have been labeled NR3A and NR3B. Incorporation of NR3 subunits modifies NMDA receptor functioning (Das et al., 1998; Chatterton et al., 2002; Matsuda et al., 2002; Matsuda et al., 2003; Smothers and Woodward, 2007). Electrophysiological studies (Chatterton et al., 2002; Matsuda et al., 2002; Matsuda et al., 2003; Smothers and Woodward, 2007) suggest that NR3 subunits can form excitatory glycine receptors when co-expressed with

NR1, although little is known about the physiological relevance of NR3 subunits in nervous system functioning.

NR1 mRNA expression develops along a caudal to rostral gradient. Regional specificity of NR1 splice variants is present at birth and remains fixed through adulthood, with the only developmental change being shifts in the density of the respective splice variants (Laurie and Seeburg, 1994; Laurie et al., 1995). NR2 mRNA expression is both developmentally and regionally regulated (Monyer et al., 1994). Prenatally, NR2B and NR2D subunits predominate, with NR2D expression levels peaking about one week after birth and NR2B levels peaking about three weeks postnatally. NR2A and NR2C subunits begin to appear near birth and increase to peak levels around the third postnatal week. After reaching peak expression, all NR2 and NR1 subunit expression decreases to adult levels (Laurie and Seeburg, 1994). NR1 subunits are found throughout the adult central nervous system (CNS); however, the expression of NR2 subtypes is more regionally restricted. NMDA receptor subunit protein expression for the most part overlaps expression patterns reported for subunit mRNAs (Petralia et al., 1994a; Petralia et al., 1994b; Portera-Cailliau et al., 1996; Wenzel et al., 1996). The NR2A subunit is the most widely expressed subtype in the adult brain. The NR2B subunit is expressed mostly in the adult cortex and the hippocampus, and NR2C is heavily expressed in the cerebellum. NR2D is moderately expressed in the adult midbrain and brainstem, with lower levels in the cortex. Expression of NR2 subunits is neuron subtype-specific within certain brain regions. In the hippocampus, for example, NR2A and NR2B are highly expressed in pyramidal neurons whereas NR2D is found in interneurons (Monyer et al., 1994).

1.2.2 NMDA receptor structure

A crystal structure of the NMDA receptor does not exist, most likely due to the difficulty in crystallizing membrane proteins, especially one as complex as the NMDA receptor. Therefore, the exact structure and location of critical determinants of receptor function are not fully understood. However, evidence exists that sheds light on the structure of NMDA receptors as well as the roles specific amino acids play in receptor functioning.

1.2.2.1 Subunit topology

The subunit topology of ionotropic glutamate receptors is conserved among NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainite receptors (Dingledine et al., 1999). The protein structure of an NMDA receptor subunit begins at the extracellular amino-terminal (N-terminal) end, contains three transmembrane regions (M1, M3, and M4) and a reentrant loop (M2) between transmembrane region M1 and M3, and ends with an intracellular carboxyl terminal tail (C-terminal) (Figure 1).

NMDA receptors contain a region between the N-terminal domain and M1 called S1, which, along with the extracellular linker region located between M3 and M4 (S2), forms the agonist binding domain (ABD). The reentrant loop formed by the M2 region lines the narrowest portion of the pore of NMDA receptors and creates the selectivity filter that allows the passage of specific ions through activated receptors. The N-site (named for the asparagine residue located near the apex of the M2 loop) of the M2 region is critical for two unique NMDA receptor properties, calcium (Ca^{2+}) permeation and block by endogenous extracellular magnesium (Mg^{2+}_o) (Burnashev et al., 1992; Kuner et al., 1996; Wollmuth et al., 1996; Wollmuth et al., 1998). The N-site of NR1 subunits strongly regulates Ca^{2+} permeability but is less involved in

mediating block by Mg^{2+}_o (Burnashev et al., 1992; Wollmuth et al., 1996; Wollmuth et al., 1998). The N + 1-site (an asparagine next to the N-site towards the C-terminal end), of NR2 subunits has been shown to have a greater involvement in mediating block by Mg^{2+}_o than regulating Ca^{2+} permeability (Burnashev et al., 1992; Wollmuth et al., 1996; Wollmuth et al., 1998). Along with forming the binding site for Mg^{2+}, the N-site is also involved in the binding of other NMDA receptor channel blockers (Dingledine et al., 1999; Kashiwagi et al., 2002). The intracellular C-terminal region of NMDA receptor subunits contains sites that modulate NMDA receptor function, such as phosphorylation sites (Tingley et al., 1997) and sites for cytoskeletal, as well as cytoskeletal linker proteins (Wyszynski et al., 1997; Ehlers et al., 1998; Lei et al., 2001).

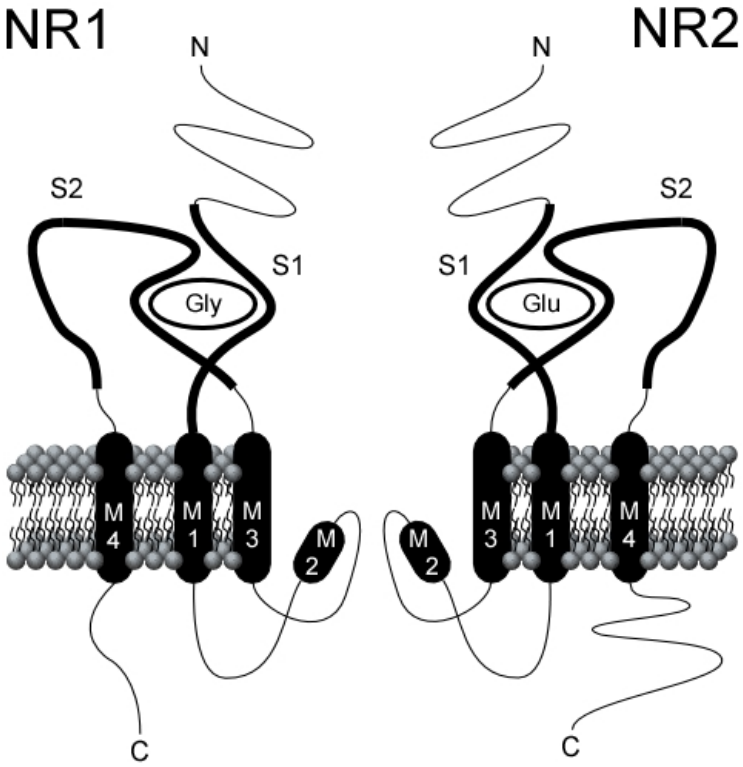

Figure 1. Topology and transmembrane structure of the NR1 and NR2 subunits of NMDA receptors. Although four subunits are likely required for a functional receptor, only two subunits are shown here for clarity. N and C designate the extracellular N-terminal and intracellular C-terminal ends of the protein, respectively. The three transmembrane regions (M1, M3, and M4) and the re-entrant pore loop (M2) are portrayed as black rounded cylinders. Residues critical in regulating ion permeability and open-channel block by Mg^{2+}_{o} as well as many channel blockers, such as memantine and ketamine, are located near the apex of the M2 region. The ligand binding pockets are depicted as the thick black lines shown N-terminal to M1 (S1) and on the M3 – M4 linker (S2). The binding of glycine (Gly) to the NR1 subunit and glutamate (Glu) to the NR2 subunit are also portrayed.

1.2.2.2 Stoichiometry

The number of NMDA receptor subunits in functional receptors is not known with certainty. Most results suggest that two NR1 and two NR2 NMDA receptor subunits form a tetrameric receptor complex (Clements and Westbrook, 1991; Laube et al., 1998; Schorge and Colquhoun, 2003). The arrangement of NR1 and NR2 subunits around the channel pore is also unknown. There is evidence that a single NMDA receptor can contain two different NR2 subunits along with NR1 subunits (Sheng et al., 1994; Chazot and Stephenson, 1997; Dunah et al., 1998). The incorporation of multiple NR2 subtypes will result in channel properties distinct from those of channels formed by NR1 subunits and one type of NR2 subunit (Wafford et al., 1993; Brimecombe et al., 1997; Vicini et al., 1998; Tovar and Westbrook, 1999; Cheffings and Colquhoun, 2000; Brickley et al., 2003).

1.2.3 NMDA receptor function

NMDA receptors possess distinctive properties that allow them to play a unique role in excitatory synaptic transmission within the CNS (Dingledine et al., 1999). Among these properties are the requirement of binding by two agonists, glutamate and glycine, for channel activation and ion flux. Once activated, NMDA receptors allow for the permeation of Ca^{2+} into neurons. Intracellular Ca^{2+} acts as an important signaling molecule inside neurons where it can activate intracellular signal transduction cascades that have wide-ranging and diverse effects on neuronal connections and viability. A third unique property, use-dependent block by endogenous Mg^{2+}_{o}, regulates the flow of Ca^{2+} through NMDA receptors.

1.2.3.1 Channel activation

Ionotropic glutamate receptor activation requires binding of ligands. The ABD is a clamshell-like structure comprised of the S1 and S2 regions of a subunit. Research on AMPA (Armstrong and Gouaux, 2000; Furukawa and Gouaux, 2003) and kainate receptors (Armstrong et al., 1998) suggests that the binding of ligand (agonist or antagonist) to the ABD results in closure of the S1S2 clamshell, which translates into movement of downstream portions of the receptor and channel opening. The binding of agonists or antagonists results in different degrees of clamshell closure that translates to the movement of the S1 and S2 domains relative to the rest of the receptor complex. Agonist binding results in clamshell closure to a degree that leads to sufficient tension on the pore-forming region to cause channel opening and allow ion flow.

Based on the conservation of topology among glutamate receptors, it is reasonable to assume that NMDA receptor activation occurs in a similar manner. The S1S2 complex of the NR1 NMDA receptor subunit binds the co-agonist glycine (Kuryatov et al., 1994; Hirai et al.,

1996; Furukawa and Gouaux, 2003), and the corresponding site on NR2 binds the co-agonist glutamate (Laube et al., 1997; Anson et al., 1998; Lummis et al., 2002). It is likely that four molecules of agonist (two glutamate and two glycine) are required for channel activation. Physiologically, aspartate and D-serine may also contribute to NMDA receptor activation by binding to the glutamate and glycine sites, respectively (Patneau and Mayer, 1990; Priestley et al., 1995; Schell et al., 1995).

1.2.3.2 Ion permeation

Activated NMDA receptors exhibit nearly equal permeability to the monovalent cations sodium (Na^+), potassium (K^+), and the less physiologically relevent cation cesium (Cs^+) (Dingledine et al., 1999). NMDA receptors are also highly permeable to the divalent cation Ca^{2+} (Burnashev et al., 1995). The influx of cations through activated NMDA receptor channels results in depolarization of the corresponding neuron. The varied and potentially deleterious effects of Ca^{2+} influx through NMDA receptors requires tight regulation of NMDA receptor-mediated transmission.

1.2.3.3 Block by endogenous Mg^{2+}_o

The binding of agonist and channel opening is not sufficient to allow ion flow through NMDA receptors. Endogenous Mg^{2+}_o enters the pore of activated NMDA receptors, binds near the N-site, and blocks the flow of ions through the channel at membrane voltages near rest (Burnashev et al., 1992; Kuner et al., 1996). Mg^{2+}_o inhibition of NMDA receptors limits the influx of Ca^{2+} through activated receptors, preventing in physiological situations, the accumulation of intracellular Ca^{2+} to toxic levels.

The extent of Mg^{2+}_o inhibition of NMDA receptors is highly dependent on membrane voltage and subunit composition (Ascher and Nowak, 1988; Monyer et al., 1994; Momiyama et al., 1996; Dingledine et al., 1999). At voltages near rest, physiological concentrations of Mg^{2+}_o inhibit a majority of NMDA receptor-mediated neurotransmission. Membrane depolarization that results from the activation of non-NMDA glutamate receptors is necessary to reduce Mg^{2+}_o inhibition of NMDA receptors and allow the flow of ions through activated channels. The type of NR2 subunit incorporated into the receptor strongly affects the degree of Mg^{2+}_o inhibition (Monyer et al., 1994; Momiyama et al., 1996). Receptors composed of NR1 with NR2A and/or NR2B subunits are more strongly inhibited by Mg^{2+}_o than receptors that contain NR1 with NR2C and/or NR2D subunits.

1.2.4 NMDA receptor pharmacology

Many drugs exist that modulate NMDA receptor function, and most drugs decrease NMDA receptor activity. Effective NMDA receptor inhibitors must be capable of preventing overactivity while preserving physiological levels of activity. Two possibilities for inhibiting overactive NMDA receptors are competitive binding by antagonists and blocking the pore of activated receptors. These two mechanisms of inhibition are associated with various advantages and disadvantages.

1.2.4.1 Competitive antagonists

Competitive antagonists of NMDA receptors work by binding at the agonist binding site. The binding of antagonist to the agonist binding site prevents the conformational changes of the receptor necessary for channel opening. The requirement of two different agonists for channel

activation offers two distinct sites at which competitive antagonists can act, the glycine binding site on the NR1 subunit and the glutamate binding site on the NR2 subunit. Kynurenic acid derivatives, such as 5, 7-dichlorokynurenic acid (5, 7-DCK), are examples of competitive antagonists at the glycine site of NMDA receptors. Phosphono derivatives of short-chain amino acids, such as 2-amino-5 phosphonopentanoic acid (AP5) and 2-amino-7 phosphonopentanoic acid (AP7), are examples of competitive antagonists at the glutamate site (Priestley et al., 1995; Dingledine et al., 1999).

Competitive antagonists, especially at the glycine site, offer a way to inhibit the functioning of all NMDA receptors, because NR1 subunits are required for functional receptors. Competitive antagonists that bind at the glutamate site, potentially could allow for subunit-specific inhibition of NMDA receptors; however, many glutamate site antagonists are not NR2 subtype selective (Lodge et al., 2002).

Inhibition by competitive antagonists is compromised in cases where a high concentration of agonist is present. This effect is useful in preserving NMDA receptor-mediated synaptic transmission where high concentrations (~1 mM) of glutamate are released that can outcompete the antagonist for binding (Lodge et al., 2002).

1.2.4.2 Channel blockers

NMDA receptor antagonism by channel blockers is use-dependent, meaning that channel activation is required for these compounds to reach their binding site and exert their effects. The N-site of NMDA receptors contributes to the binding site of several NMDA receptor channel blockers, including Mg^{2+}_{o} (Kashiwagi et al., 2002). NMDA receptor inhibition by channel blockers is voltage-dependent, since their binding site is located within the membrane voltage field. Voltage-dependent inhibition results in a decrease in a channel blocker's ability to inhibit

NMDA receptor activity as membrane voltage depolarizes (MacDonald et al., 1987; MacDonald et al., 1991; Parsons et al., 1993; Parsons et al., 1995; Parsons et al., 1996; Blanpied et al., 1997; Aracava et al., 2005; Wrighton et al., 2008).

A channel blocker can either prevent the closing of the channel while bound, or it can allow for channel closure around the bound blocker. The differential effects of blockers on channel gating have led to the classification of channel blocking drugs as either sequential (foot-in-the-door) blockers or trapping blockers, respectively.

Sequential channel blockers are not unique to NMDA receptors and have been described for other ligand-gated ion channels (Neher and Steinbach, 1978). The binding of a sequential channel blocker within the channel of activated receptors inhibits not only ion flow but also channel closure (Benveniste and Mayer, 1995; Antonov and Johnson, 1996). A representative NMDA receptor sequential channel blocker is 9-aminoacridine (9-AA). The mechanism responsible for stabilizing the open state of NMDA receptor channels while sequential blockers are bound is not completely understood.

Trapping channel blockers bind within the channel of activated NMDA receptors and allow subsequent channel closure and dissociation of agonists, an effect that traps the drug within the pore of the receptor. The rebinding of agonist and channel activation allows unbinding of the trapped blocker and its escape from the open channel (Huettner and Bean, 1988; Lerma et al., 1991; MacDonald et al., 1991; Parsons et al., 1993; Parsons et al., 1995; Parsons et al., 1996; Blanpied et al., 1997; Chen and Lipton, 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Mealing et al., 1999; Sobolevsky, 1999; Mealing et al., 2001; Kashiwagi et al., 2002; Bolshakov et al., 2003; Wrighton et al., 2008). There are differences in the degree to which trapping blockers remain bound to closed channels. Full trapping channel blockers

(such as the dissociative anesthetics ketamine and PCP) are compounds that, once trapped within closed NMDA receptors, cannot be released until subsequent channel opening allows escape back to the extracellular environment (Huettner and Bean, 1988; Lerma et al., 1991; Mealing et al., 1999; Mealing et al., 2001; Bolshakov et al., 2003). Partial trapping channel blockers (such as the amino-adamantane derivative memantine) are capable of dissociating from a fraction of NMDA receptors before subsequent channel activation allows open channel escape (Blanpied et al., 1997; Chen and Lipton, 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Mealing et al., 1999; Mealing et al., 2001; Bolshakov et al., 2003). The underlying mechanism through which partial trapping channel blockers prevent being trapped is unknown.

1.3 NMDA RECEPTOR ROLE IN PHYSIOLOGY AND PATHOLOGY

1.3.1 NMDA receptor role in synaptic transmission

NMDA receptor-mediated neurotransmission is tightly regulated. NMDA receptors are coincidence detectors because of the requirement of presynaptic glutamate release and postsynaptic depolarization for channel activation and Mg^{2+}_o unblock to occur (Collingridge et al., 1988; McBain and Mayer, 1994; Albensi, 2007). Postsynaptic depolarization typically arises from activation of other members of the glutamate receptor family, mainly AMPA receptors.

1.3.1.1 Learning and memory

Alteration in the strength of synaptic connections is believed to be a biophysical mechanism underlying learning and memory. Changes in the strength of a synapse can be expressed as

either an increase or a decrease in the ability of a synapse to transmit information. A long-lasting increase in the efficacy of a synapse is termed long-term potentiation (LTP) and a long-lasting decrease long-term depression (LTD) (Bliss and Collingridge, 1993; Rison and Stanton, 1995; Bear and Abraham, 1996; Riedel et al., 2003). NMDA receptor activation has been shown to be critical in the expression of many forms of LTP and LTD at excitatory synapses (Malenka and Bear, 2004).

For LTP induction, the intensity of postsynaptic activity must be strong enough to initiate the cascade of events that will strengthen the connection between the pre- and post-synaptic neuron. Cooperativity, associativity, and input-specificity are three properties commonly found to be associated with LTP. Cooperativity refers to the convergence and spatial/temporal pairing of signals that arise from many fibers. Each signal alone is not intense enough to yield LTP; however, the combined activity of all the fibers converging on a postsynaptic site can surpass the threshold of signal strength required to result in LTP. The ability of a weak signal to be potentiated, if its activity is paired with a strong stimulus at a separate but connected site, is called associativity. Input-specificity, which is not a universal property of LTP, refers to the expression of LTP being localized to the activated synapse and not induced at other synapses of the same neuron (Bliss and Collingridge, 1993; Rison and Stanton, 1995).

The requirements for the induction of LTP can be linked to the unique properties of NMDA receptors and the organization of the synapse. The presynaptic release of glutamate will activate glutamate receptors within the postsynaptic membrane. Low-intensity activity results in the activation and passage of ions through AMPA receptors, while NMDA receptor activity is inhibited by Mg^{2+}_{o}. Higher-intensity activity results in greater AMPA receptor-mediated current and depolarization of the postsynaptic site, relieving Mg^{2+}_{o} block of NMDA receptors. The

requirement for presynaptic glutamate release and postsynaptic depolarization intense enough to reduce Mg^{2+}_o block of NMDA receptors accounts for the associative and cooperative properties of LTP. The activation of postsynaptic NMDA receptors results in the influx of Ca^{2+}, which activates various second messenger systems localized to the region of the activated postsynaptic site, accounting for the input-specificity of LTP. The activation of second messengers by Ca^{2+} influx results in synaptic changes, such as increasing the flow of ions through postsynaptic receptors, that contribute to the expression of LTP (Bliss and Collingridge, 1993; Rison and Stanton, 1995).

While high levels of synaptic activity are required for LTP induction, lower levels of activity can produce LTD (Malenka and Bear, 2004). NMDA receptors have been implicated in the induction of some forms of LTD, since inhibition of NMDA receptors has been shown to prevent LTD induction (Dudek and Bear, 1992; Thiels et al., 1996). Like LTP, it appears that increases in intracellular Ca^{2+} concentrations, likely through activated NMDA receptors, is critical for inducing LTD (Mulkey and Malenka, 1992). The increase in postsynaptic Ca^{2+} concentration resulting from LTD-inducing stimuli is hypothesized to activate a cascade of phosphatases that decreases the flow of ions through postsynaptic receptors (Mulkey et al., 1993). Thus, it appears that the magnitude of Ca^{2+} entering the postsynaptic cell through NMDA receptors is a crucial factor in determining the direction of change in synaptic strength (Mulkey and Malenka, 1992).

1.3.2 Pathological consequences of NMDA receptor activity

NMDA receptor blockade can increase the vulnerability of neurons to cell death, especially early in development (Ikonomidou et al., 1999; Ikonomidou et al., 2000; Hardingham and Bading,

2003). Administration of NMDA receptor antagonists to perinatal rats has been reported to increase neuronal death (Ikonomidou et al., 1999; Lipton and Nakanishi, 1999; Ikonomidou et al., 2000). The increase in neuronal death was observed for various NMDA receptor antagonists (MK-801, PCP, ketamine, memantine, or CPP). Thus, it is suggested that some level of NMDA receptor-mediated activity is required for cell growth and survival (Ikonomidou et al., 1999; Ikonomidou et al., 2000; Hardingham and Bading, 2003). The exact mechanism underlying the requirement of NMDA receptor activation for neuronal survival is unknown. One suggested mechanism is that excessive inhibition of NMDA receptors can lower intracellular Ca^{2+} levels. This depletion of intracellular Ca^{2+} will lower the activity of Ca^{2+}-activated signaling molecules whose activity promotes cell survival (Ikonomidou et al., 2000; Hardingham and Bading, 2003).

Excessive activation of NMDA receptors and the resultant Ca^{2+} influx can also result in neuronal cell death. Thus, high Ca^{2+} permeability of NMDA receptors appears responsible for their influence on the health and survival of neurons. Pro-survival or pro-death pathways can be activated depending on the source as well as degree of Ca^{2+} influx into a cell. The exact contribution of different Ca^{2+} sources to the onset of neuronal cell death is still undetermined (Hardingham and Bading, 2003).

1.3.2.1 Excitotoxic cell death

Excessive glutamate release, resulting from events such as head trauma, can overwhelm glutamate transporters and cause hyperactivation of glutamate receptors. Intense activation of NMDA receptors leads to accumulation of Ca^{2+} in neurons that surpasses the ability of endogenous regulators of Ca^{2+} sequestration. High concentrations of free intracellular Ca^{2+} can initiate many downstream signaling cascades that can harm the health of neurons. The consequences of these signaling cascades include alteration of mitochondrial function,

overactivation of enzymes that produce reactive oxygen species (ROS), reorganization of the cytoskeleton, and activation of genetic cell death signals (Choi, 1988; Lodge et al., 2002; Hardingham and Bading, 2003; Arundine and Tymianski, 2004; Albensi, 2007). Triggering of these cell death pathways contributes to the pathology of both acute insults and more chronic neurodegenerative disorders.

1.3.2.2 Neuropsychiatric and neurodegenerative disorders

While severe insults can rapidly initiate NMDA receptor-mediated cell death, mild but chronic alterations in NMDA receptor functioning are hypothesized to contribute to many neuropsychiatric (such as schizophrenia) and neurodegenerative (such as Alzheimer's and Parkinson's disease) disorders. NMDA receptor malfunctioning has also been implicated in other diseases, such as neuropathic pain, epilepsy, and glaucoma (Lodge et al., 2002). Although involved in many disease states, the focus of this dissertation will be limited to NMDA receptor involvement in schizophrenia and Alzheimer's disease.

Schizophrenia is a severe cognitive disorder that typically begins in early adulthood and lasts throughout an individual's lifetime. The symptoms of schizophrenia have typically been separated into three categories: positive, negative, and cognitive. The positive symptoms include hallucinations, paranoia, and agitation, which are believed to result from increases in normal CNS functioning. The negative symptoms of schizophrenia represent those that reflect a decrease in CNS functioning, such as dulled emotional responses and social withdrawal. Disorientation and conceptual disorganization reflect the cognitive symptoms (Kilts, 2001; Lodge et al., 2002; Lindsley et al., 2006).

Although alterations in the functioning of several neurotransmitter systems have been implicated in schizophrenia, much attention has focused on the involvement of the dopaminergic

and glutamatergic systems. The dopamine hypothesis proposes that excessive dopamine receptor activity (especially D_2 receptors) underlies the positive symptoms of schizophrenia (Seeman, 2002; Stone et al., 2007). This hypothesis is based on the clinical observation that pharmacological dopamine receptor antagonists are effective in alleviating the positive symptoms of schizophrenia (Seeman, 2002; Stone et al., 2007). The NMDA receptor hypofunction hypothesis arose from the observation that NMDA receptor antagonists, such as PCP and ketamine are capable of reproducing a wide array of symptoms characteristic of schizophrenia in healthy individuals as well as worsen symptoms in already afflicted patients (Lindsley et al., 2006; Stone et al., 2007). The NMDA receptor hypofunction hypothesis claims that decreased NMDA receptor-mediated activity is critically involved in the pathology of schizophrenia (Lindsley et al., 2006; Stone et al., 2007).

The glutamatergic and dopaminergic systems interact and modulate the functioning of each other (Jedema and Moghddam, 1996; Kiyatkin and Rebec, 1996; Verma and Moghaddam, 1996; Moghaddam et al., 1997; Adams and Moghaddam, 1998; Takahata and Moghaddam, 1998; Carr and Sesack, 2000; Sesack et al., 2003; Stone et al., 2007). Dopamine modulates the functioning of glutamatergic projection neurons in the cortex and the hippocampus, areas associated with cognition and learning (Kiyatkin and Rebec, 1996; Stone et al., 2007). Firing of glutamatergic neurons also affects the activity of dopaminergic neurons (Jedema and Moghddam, 1996; Verma and Moghaddam, 1996; Moghaddam et al., 1997; Adams and Moghaddam, 1998; Takahata and Moghaddam, 1998). Ketamine administration to rats resulted in an increase of dopamine release in the prefrontal cortex (Verma and Moghaddam, 1996; Moghaddam et al., 1997), demonstrating a functional link between the dopaminergic hypothesis and NMDA receptor hypofunction hypothesis of schizophrenia. However, it is still unclear

whether alterations in the dopaminergic or glutamatergic neurotransmitter systems are the first step in the onset of schizophrenic pathology.

Alzheimer's disease is a progressive form of dementia that affects the elderly. Pathological hallmarks of Alzheimer's disease are the presence of senile plaques and neurofibrillary tangles, and a loss of synaptic connections (Butterfield and Pocernich, 2003). Senile plaques are comprised of amyloid β-peptide (Aβ) deposits, which are suggested to be associated with the neurodegeneration observed in Alzheimer's disease (Varadarajan et al., 2000). Aβ is produced by enzymatic cleavage of the membrane associated protein amyloid precursor protein (APP). Hyperphosphorylation of the microtubule-associated protein tau forms neurofibrillary tangles by disrupting microtubule assembly. Disruption of the microtubule system results in cellular dysfunctions contributing to death (Alonso et al., 1997; Varadarajan et al., 2000; Chohan and Iqbal, 2006).

Conventional treatments for Alzheimer's disease have been aimed at enhancing cholinergic neurotransmission by inhibiting cholinesterase (an enzyme that degrades acetylcholine) activity. In fact, it has been suggested that cholinergic transmission controls APP cleavage, Aβ plaque formation, and tau phosphorylation (Schliebs and Arendt, 2006). However, it is still unclear whether a deficit in cholinergic transmission is the initiator or a result of Alzheimer's disease. Much like the proper functioning of the dopaminergic and glutamatergic systems is relevant in the pathophysiology of schizophrenia, it is likely that multiple neurotransmitter systems are involved in Alzheimer's disease. Evidence linking NMDA receptors to learning and memory as well as excitotoxic cell death suggests that the glutamatergic system is likely to be highly involved in Alzheimer's disease (Albensi et al., 2004). Further supporting NMDA receptor involvement in the pathology of Alzheimer's disease

is evidence that NMDA receptor activity stimulates Aβ production (Butterfield and Pocernich, 2003) and NMDA receptor-dependent synaptic transmission is suppressed by Aβ (Butterfield and Pocernich, 2003; Kamenetz et al., 2003). Aβ also causes a loss of excitatory terminals in the cortex (Bell and Claudio Cuello, 2006) and NMDA receptor internalization (Snyder et al., 2005). The ability of the NMDA receptor antagonist memantine to slow the cognitive decline of moderate-to-severe Alzheimer's disease also links NMDA receptor activity to the disease (Parsons et al., 1999; Farlow, 2004; Lipton, 2004a; Winblad et al., 2007).

1.3.3 Behavioral effects of NMDA receptor inhibition

Increased NMDA receptor activity is implicated in a variety of disease states; however, pharmacological attempts at preventing pathological NMDA receptor overactivation have generally been less than promising. Among other problems, many NMDA receptor antagonists are associated with debilitating psychotomimetic side-effects. While therapeutically this side-effect profile has hindered the clinical utility of many NMDA receptor antagonists, it has also greatly furthered our understanding of diseases characterized by NMDA receptor hypofunction, especially schizophrenia.

1.3.3.1 Human effects

The effects of the dissociative anaesthetics, PCP and ketamine, in humans have been well characterized. The main action of these drugs is suggested to be use-dependent inhibition of NMDA receptor-mediated neurotransmission (Bormann, 1989; Kornhuber et al., 1989; Yamamura et al., 1990; Witt et al., 2004). PCP and ketamine have been crucial tools in the study of schizophrenia. Human psychopharmacological research has shifted towards ketamine rather

than PCP due to the lower abuse potential of ketamine and its shorter plasma half-life which allows for greater control of plasma levels (Krystal et al., 2003). The use of ketamine to model schizophrenia in humans is based on a variety of observations. The strongest support comes from ketamine's ability to transiently mimic the symptoms of schizophrenia in healthy adults (Newcomer et al., 1999; Krystal et al., 2003) as well as transiently worsen symptoms in individuals already afflicted with schizophrenia (Lahti et al., 1995; Malhotra et al., 1997; Krystal et al., 2003).

Although ketamine has been widely accepted as a pharmacological model of schizophrenia, some discrepancies exist between the effects of ketamine and the disorder. Ketamine administered to schizophrenic patients in remission resulted in psychotic relapse. The symptoms exhibited during the relapse were typical for a given patient; however, these symptoms were not ameliorated by pretreatment with the typical antipsychotic haloperidol (Krystal et al., 1999a). Another difference between ketamine's effects and the symptomatic profile of schizophrenia is the ability of ketamine to produce sedative and euphoric effects similar to alcohol (Krystal et al., 2003).

1.3.3.2 Ketamine as an animal model of schizophrenia

Administration of the NMDA receptor antagonists PCP, ketamine, or MK-801 leads to behaviors in rat that have been correlated to the symptoms of schizophrenia in humans (Lipska and Weinberger, 2000; Kilts, 2001; Tordjman et al., 2007). Psychomotor agitation in humans is correlated with increased locomotor activity in rats (Hetzler and Wautlet, 1985; Danysz et al., 1994; Koros et al., 2007). Also both schizophrenic humans and rats administered dissociative anaesthetics exhibit spatial working memory deficits (Verma and Moghaddam, 1996; Jackson et

al., 2004), stereotypic behavior, and decreased social behavior (Danysz et al., 1994; Lipska and Weinberger, 2000; Kilts, 2001; Koros et al., 2007; Tordjman et al., 2007).

1.3.4 NMDA receptors as therapeutic targets

Some NMDA receptor antagonists demonstrate potential in alleviating the symptoms of many disorders; however, their usefulness has been hindered by the debilitating side-effects discussed previously. Surprisingly, despite the psychotomimetic side-effects elicited by NMDA receptor antagonists in healthy human adults, these side-effects are much less severe in children.

Despite the intolerability of many NMDA receptor antagonists, the antagonist memantine is reported to be generally safe. Furthermore, memantine has clinical value in its ability to slow the progressive cognitive decline associated with moderate-severe Alzheimer's disease (Witt et al., 2004).

1.3.4.1 Memantine and Alzheimer's disease

Although generally well tolerated (Parsons et al., 1999; Rogawski and Wenk, 2003; Witt et al., 2004; Sonkusare et al., 2005), there have been reports of probable Alzheimer's patients experiencing visual hallucinations and agitation about a week after having memantine (5 mg/day) added to their combination treatment (Monastero et al., 2007). However, controlled studies of patients with moderate to severe Alzheimer's disease have shown that the overall incidence of adverse reactions to memantine was not different than the incidence of these reactions to placebo (Mobius, 2003).

Memantine administered to rats induced behavioral effects that were similar, yet less prominent, than those induced by ketamine. The behavioral effects included increased horizontal

activity, decreased rearings, and decreased social interaction (Danysz et al., 1994; Koros et al., 2007). It should also be mentioned that the doses of memantine administered to the rats in these studies was much higher than those given to individuals receiving typical treatment regimens.

2.0 BINDING AT SUPERFICIAL SITE ON NR1/2A NMDA RECEPTORS CONTRIBUTES TO PARTIAL TRAPPING OF MEMANTINE

2.1 ABSTRACT

Memantine lacks the behavioral side-effects of conventional NMDA receptor channel blockers, such as ketamine and phencyclidine (PCP) and slows the progressive cognitive decline associated with Alzheimer's disease. Memantine and ketamine inhibit NMDA receptors with similar affinity and kinetics at a site that overlaps the binding site for Mg^{2+}_{o}. A prominent mechanistic difference between inhibition by memantine or ketamine is the degree to which they are trapped within the channel of closed NMDA receptors. Here, we have investigated the mechanism underlying partial trapping of memantine by recombinant NR1/2A NMDA receptors. We measured a time course ($\tau = 0.78 \pm 0.29$ s) for the dissociation of memantine from NR1/2A receptors; this time course describes the development of partial trapping. Our data argue against open channel escape of memantine during partial trapping development. We and others have measured memantine inhibition of NMDA receptors in the absence of agonist, an effect that has not been observed with ketamine. Based on our observations we conclude that partial trapping of memantine results from competitive binding of memantine at a superficial "non-trapping" site and the deep "trapping" site. Memantine binding at the superficial site may, by causing partial trapping, contribute to memantine's unique therapeutic utility and clinical effectiveness.

2.2 INTRODUCTION

Despite differences in tolerability, memantine and ketamine are both activity-dependent NMDA receptor channel blockers with comparable affinities and kinetics. These drugs require agonist binding and channel opening for access to their binding site (Chen et al., 1992; Parsons et al., 1995; Blanpied et al., 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Mealing et al., 1999; Bolshakov et al., 2003). Channel closure and agonist dissociation can occur while either drug is bound, effectively trapping the drug within the receptor's channel until subsequent agonist binding again allows channel activation and blocker dissociation. The ability of channel blockers to enter into this trapped state varies among different NMDA receptor trapping channel blockers. Ketamine has been classified as a "full trapping" channel blocker, because all (or nearly all) molecules bound to NMDA receptors following dissociation of agonist remain bound until further channel activation (Mealing et al., 1999). Memantine has been classified as a "partial trapping" channel blocker because a fraction of memantine molecules bound to NMDA receptors immediately after agonist removal has been shown to unbind before channels are subsequently activated by reapplication of agonist (Blanpied et al., 1997; Chen and Lipton, 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Mealing et al., 1999). Hypotheses have been put forth to explain how the non-trapped fraction of partial trapping blockers dissociates from NMDA receptors (Blanpied et al., 1997; Mealing et al., 2001; Bolshakov et al., 2003); however, the mechanism remains unclear.

In some studies that examined the mechanism responsible for the varied degrees of trapping of NMDA receptor channel blockers, partial trapping compounds were hypothesized to leave the channel through an open state (Blanpied et al., 1997; Bolshakov et al., 2003). Proposed "open-channel escape" mechanisms include the ideas that memantine binding increases agonist

affinity and gating transitions into the open state (Blanpied et al., 1997), and that partial trapping compounds bind at a second site that prevents channel closure (Bolshakov et al., 2003). Mealing et al. (2001) proposed the presence of a second "trapping gate" (distinct from the gate that regulates ion permeation) that closes slowly compared to the unbinding rate of partial trapping compounds. However, this same group also reported that differences in the degree of trapping exist among use-dependent NMDA receptor antagonists that have similar binding and unbinding kinetics, arguing against a compounds unbinding kinetics being the sole contributor to partial trapping (Mealing et al., 1999).

Although capable of explaining how partial trapping can occur, these hypotheses are not entirely consistent with experimental observations. A problem associated with open-channel escape models of partial trapping is that one would expect to observe current flowing through receptors that have released blocker upon the removal of agonist and blocker before channels have had the time to close. This "tail current", which is observed for sequential (foot-in-the-door) blockers that do not allow channel closure while bound (Benveniste and Mayer, 1995), is rarely observed with memantine (Blanpied et al., 1997). These observations suggest that minimal memantine dissociation occurs through open channels.

Memantine passage through a lipophilic pathway is an alternative hypothesis to open-channel escape; however, previous studies have shown no correlation between the lipophilicity of a compound and its degree of trapping (Mealing et al., 1999; Mealing et al., 2001). The observation that trapping does not continually decrease during extended wash periods (Blanpied et al., 1997) also argues against a lipophilic pathway for blocker escape.

A characteristic of NMDA receptor inhibition by memantine that may contribute to partial trapping and deserves further evaluation is the possibility that memantine can inhibit

NMDA receptors by binding at two sites. The principal binding site for memantine, as well as ketamine, is located deep within the voltage field of NMDA receptor channels and is only accessible to channel blockers upon receptor activation. Previous studies suggest the presence of a second binding site for memantine; however, the location and characteristics of the second site are less clear than for the principal binding site (Blanpied et al., 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Chen and Lipton, 2005). Evidence places this second site shallower within the voltage field of NMDA receptors than the trapping site (Blanpied et al., 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Chen and Lipton, 2005). Blanpied et al. (1997) showed that memantine can bind to and inhibit NMDA receptors at a second "superficial" site that is accessible in the absence of agonists. It is unknown whether or not ketamine can bind to the superficial site. A careful characterization of the superficial site may help further our understanding NMDA receptor functioning.

2.3 MATERIALS AND METHODS

2.3.1 Cell culture and transfection

Experiments were performed on the human embryonic kidney (HEK) 293T cell line. Cells were enzymatically dissociated, plated onto glass coverslips coated with poly-D-lysine and collagen (Sigma, St. Louis, MO), and incubated at 37º C for 24 hours prior to transfection. Transfection of the NR1-1a (GenBank accession number (ACCN) X63255, in pcDM8) and NR2A (ACCN M91561, in pcDM8) NMDA receptor subunit cDNAs, along with eGFP cDNA for identification of successfully transfected cells, was performed with Lipofectamine (Invitrogen Corporation,

Carlsbad, CA) at a ratio of 1 NR1 : 3 NR2A : 6 eGFP. 200 μM DL-2-amino-5-phosphonopentanoic acid (DL-APV, Sigma, St. Louis, MO) was added to culture medium at the end of transfection to limit excitotoxic activation of expressed NMDA receptors.

2.3.2 Solutions

Solutions were prepared from concentrated stock solutions. The external bath solution (Normal Ringers Solution) contained (in mM): 140 NaCl, 2.8 KCl, 1 $CaCl_2$, and 10 HEPES. 4-fold lower extracellular cation solution (low $[ion]_o$ Ringers) consisted of (in mM): 35 NaCl, 0.7 KCl, 0.25 $CaCl_2$, 10 HEPES. External solutions were adjusted to a pH of 7.20 ± 0.05 with 6.0 N NaOH, and osmolality adjusted to 290 ± 10 mmol/kg with sucrose. Internal solution contained (in mM): 125 CsCl, 10 BAPTA, and 10 HEPES. The pH of the internal solution was adjusted to 7.20 ± 0.05 with CsOH and osmolality was 275 ± 10 mmol/kg. A frozen aliquot of internal solution was thawed for each day of experiments. Correction for the junction potential (measured as -6 mV) was applied to all data.

2.3.3 Electrophysiology

Whole-cell patch-clamp recordings were performed on transiently transfected HEK293T cells expressing NR1/2A receptors. Recording electrodes were pulled from standard–walled borosilicate glass capillary tubes with filaments (Warner Instruments, Hamden, CT). Electrode tips were heat polished and had a resistance between 2 to 6 MΩ. Series resistance was compensated 80 – 95 % in all experiments. Whole-cell current responses were recorded with an Axopatch-1D amplifier (Molecular Devices, Sunnyville, CA), printed on a thermal arraycorder

(Graphtech Corp., Irvine, CA) for monitoring of data quality during experiments, and stored on VHS tape for additional analysis.

A seven-barrel, gravity-fed fast perfusion system (Blanpied et al., 1997) was used for solution application to patch-clamped cells. Currents were activated by application of 10 μM NMDA and 10 μM glycine. Recordings were made at -66 mV unless otherwise stated. For experiments testing the effect of a depolarizing voltage step on the fractional recovery of memantine, a voltage step to +54 mV for 5 s was applied using pClamp 9.2 (Molecular Devices, Sunnyville, CA).

The time constant for solution exchange was determined by measuring the decay time constant of NMDA receptor-mediated responses when solutions were switched from Normal Ringers Solution containing 10 μM NMDA and 10 μM glycine to a solution where 140 mM NMDG replaced NaCl. The exchange time constant determined through this method was measured as ~16 ms. Therefore, the shortest solution application we used was 0.1 s, which was greater than 5x our measured solution exchange time constant, allowing us to be sure we have achieved at least ~98 % of solution exchange. We were also concerned with the time needed for high concentrations of memantine or ketamine to diffuse away from the cell we were recording from. We measured the decay time constant of current responses upon removal of 10 mM NMDA and 10 μM glycine, to determine the time necessary to allow complete diffusion of this high NMDA concentration (relative to its EC_{50}) away from the cell. These measurements suggested complete diffusion of this high NMDA concentration occurred within 0.4 s. Therefore, 0.4 s is the shortest solution application used when applying relatively high concentrations (compared to their IC_{50} values) of memantine or ketamine to our cells.

2.3.4 Analysis

Data were played back from VHS tapes and low-pass filtered at 2.5 kHz through a Bessel filter and digitized using a Digidata 1200 or 1440A (Molecular Devices, Sunnyville, CA) at a sampling rate of 5 kHz or 400 Hz and saved using pClamp 9.2 software (Molecular Devices, Sunnyville, CA). Data were again filtered at 100 Hz and decimated by a factor of 10 using Clampfit 9.2 (Molecular Devices, Sunnyville, CA) for further analysis.

Current measurements of interest are indicated in Figure 2 and defined below. Peak current responses to the initial agonist application (I_p) were measured by subtracting baseline current from peak current. Baseline currents were calculated by averaging current within a 500 ms window. Peak currents were calculated by averaging current within a 50 ms window beginning at the time peak current was observed. The difference between the time of barrel movement and the center of the time window used to measure peak current was termed t_P and will be used below. Steady-state current responses (I_{SS}, steady-state current response to initial agonist application; I_{BSS}, steady-state current response in the presence of agonist and blocker; and I_{SS2}, steady-state current response in the presence of agonist after recovery from block) were measured by subtraction of baseline current from the average steady-state current within a 500 ms window. Data were rejected if the I_{SS2} did not recover to 60% of the previous I_{SS}. I_B is the initial current jump activated by reapplication of agonist, and exceeds I_{BSS} as a result of partial trapping. The time after barrel movement when I_B was measured equaled t_P (50 – 250 ms after barrel movement). To determine I_B, a single or double exponential was fit to a 1 s window during the current response activated by the second agonist application, beginning at a time after barrel movement equal to t_P. Baseline current was then subtracted from current value of the exponential fit measured at t_P after barrel movement to yield I_P.

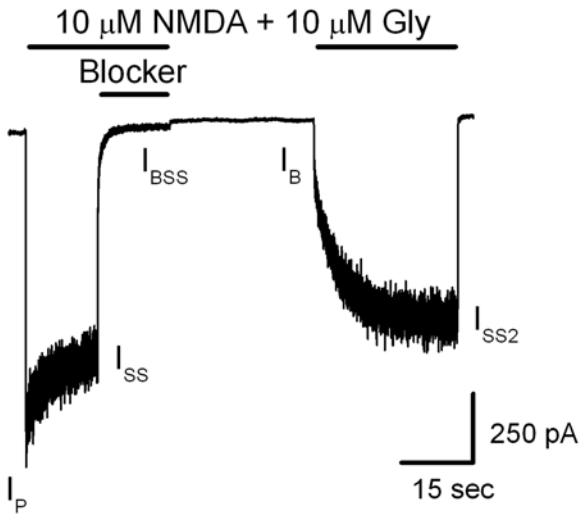

Figure 2. Double-pulse protocol. Sample trace shows the double-pulse protocol used for measuring the fractional recovery of NR1/2A NMDA receptors from inhibition by memantine or ketamine (50 μM memantine was used here). The lines above the current trace in this and subsequent figures show time of agonist and blocker applications. Current measurements of interest are indicated in the figure and defined in methods.

The residual current during inhibition by each blocker was calculated with the equation:

$$\text{Residual Current} = I_{BSS}/I_{SS} \qquad (1)$$

where I_{BSS} and I_{SS} represent the measurements shown in Figure 2, although the protocol shown in Figure 3A, B was used to measure Residual Current.

Concentration-inhibition curves for the deep site were fit using the equation:

$$I_{BSS}/I_{SS} = 1 / (1 + [B]^{n}H / IC_{50}^{n}H) \quad (2)$$

where [B] is the blocker concentration, and n_H is the Hill coefficient.

The fractional current recovery as a result of blocker unbinding between the blocker plus agonist and the agonist application in the double-pulse protocol was calculated by the equation:

$$\text{Fractional Recovery} = [(I_B - I_{BSS})/I_{SS2}]/(I_P/I_{SS}) \quad (3)$$

I_P/I_{SS} was included in the equation to account for NMDA receptor recovery from desensitization between agonist applications.

Time constants for the development of partial trapping and dissociation from the superficial site were determined by a single exponential fit of fractional recovery as a function of time (see Figure 4C) with the equation:

$$\text{Fractional Recovery} = y_0 + Ae^{-t/\tau} \quad (4)$$

where y_0 is the fractional recovery at time = 0, A is the amplitude of the exponential, t is the wash duration, and τ is the time constant.

The superficial site current recovery resulting from blocker unbinding during a wash between blocker application and agonist application (Figure 7) was calculated with the equation:

$$\text{Superficial Site Recovery} = (I_B/I_{SS2})/(I_P/I_{SS}) \quad (5)$$

where I_B, I_{SS2}, I_P, and I_{SS} represent the measurements shown in Figure 2, although the protocol used is shown in Figure 7A, B.

Concentration-inhibition curves for the superficial site were fit using the equation:

$$I_{BSS}/I_{SS} = C_1 + (1 - C_1) / [(1 + [B]^{n}H \ / IC_{50}^{n}H)] \quad (6)$$

where C_1 represents the minimal fraction of residual current when blocker is bound at the superficial site.

Where appropriate, two-tailed Student's t-test (* $p < 0.05$ for between group comparisons) or one-way ANOVA with Bonferroni *post-hoc* comparisons (# $p < 0.05$ for within group comparisons) were used.

2.4 RESULTS

2.4.1 Time course of blocker dissociation from NR1/2A receptors in the absence of agonist

We performed whole-cell voltage-clamp recordings on HEK293T cells expressing recombinant NR1/2A receptors to examine the mechanism of partial trapping. Concentration-inhibition curves for memantine and ketamine were measured (Figure 3A-C), and the resultant IC_{50} values were 1.25 ± 0.04 μM and 0.35 ± 0.01 μM, respectively. These values are similar to those reported previously (Parsons et al., 1995; Blanpied et al., 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Mealing et al., 1999; Kashiwagi et al., 2002). We chose to study partial trapping of memantine at 50 μM, a concentration consistent with that used in previous work (Blanpied et al., 1997), and wanted to chose a ketamine concentration so that we had similar residual current in ketamine as well as in memantine. Based on our IC_{50} measurements we calculated that 15 μM ketamine yields residual current comparable to that observed with 50 μM memantine (Figure 3C).

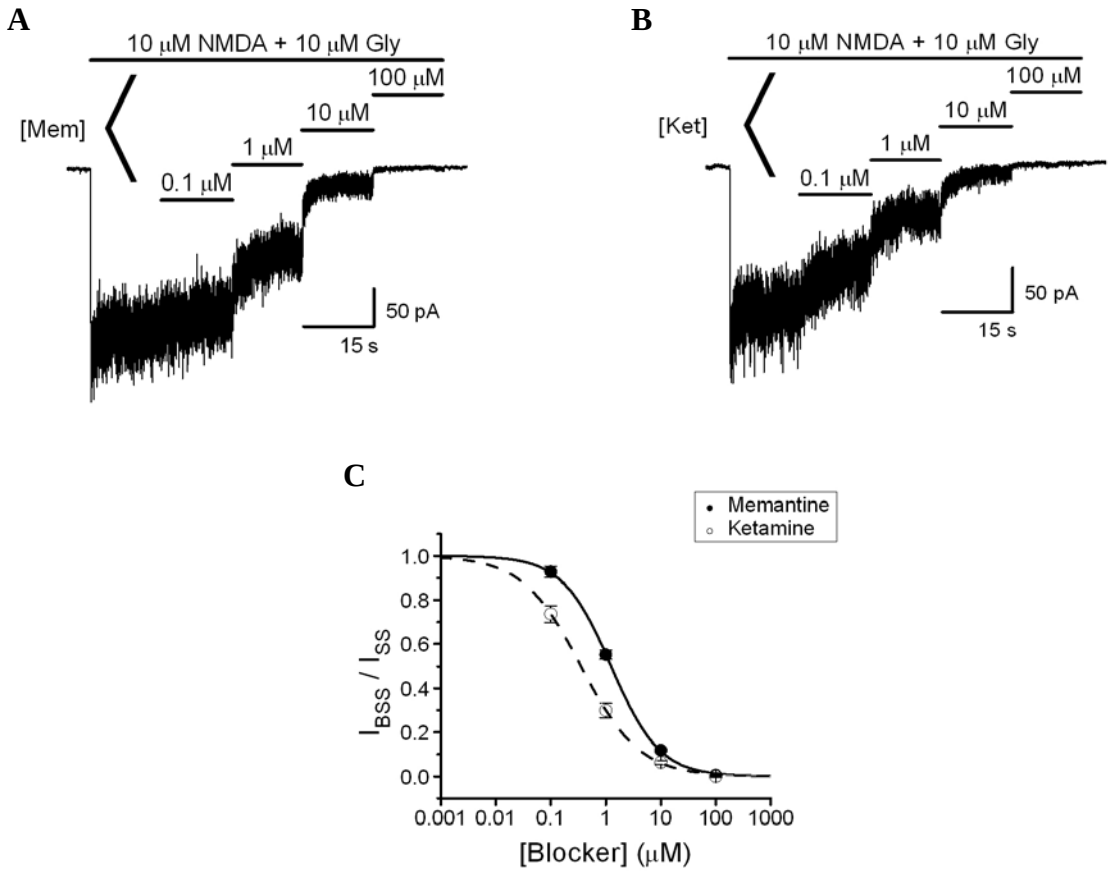

Figure 3. NMDA receptor inhibition by memantine and ketamine. A and B, Representative current traces recorded at -66 mV from transfected HEK293T cells demonstrating the concentration dependence of NR1/2A receptor inhibition by memantine (A) and ketamine (B). C, Memantine (filled circles) and ketamine (open circles) concentration-inhibition curves. Solid and dashed lines are fits (eqn (2)) to the memantine (n = 4) and ketamine (n = 4) data, respectively.

We measured the time course for the dissociation of non-trapped memantine from NR1/2A receptors in the absence of agonist that results in partial trapping. A double-pulse protocol was used (Figure 4A, B). The interval between the removal of agonist and memantine and the subsequent application of agonist to our cells was varied, which allowed us to measure the development of fractional recovery from the trapped state. The briefest wash duration used here (0.1 s) was chosen based on our measurements of solution exchange time (Methods), so we could be sure that memantine was no longer present in the solution surrounding the cell when agonist was reapplied. Our data show that the fractional recovery of memantine inhibition developed with a time constant of 0.78 ± 0.29 s (Figure 4A, C). The fractional recovery of memantine inhibition was significantly different from the residual current measured, when memantine was applied in the presence of agonist, across the wash durations tested ($p < 0.05$, ANOVA). ANOVA followed by Bonferroni *post-hoc* tests show that the fractional recovery of memantine inhibition at all wash durations tested was significantly greater than the residual current measured in the presence of memantine and agonist (p's < 0.05). A time constant of 0.58 ± 0.30 s was measured when this experiment was performed with ketamine (Figure 4B, C). There was also a significant effect of wash duration on fractional recovery of ketamine inhibition ($p < 0.05$, ANOVA). Although these results suggest that ketamine exhibits slight partial trapping, fractional recovery from inhibition was significantly lower with ketamine than with memantine at all wash durations tested (p's < 0.05, Figure 4C).

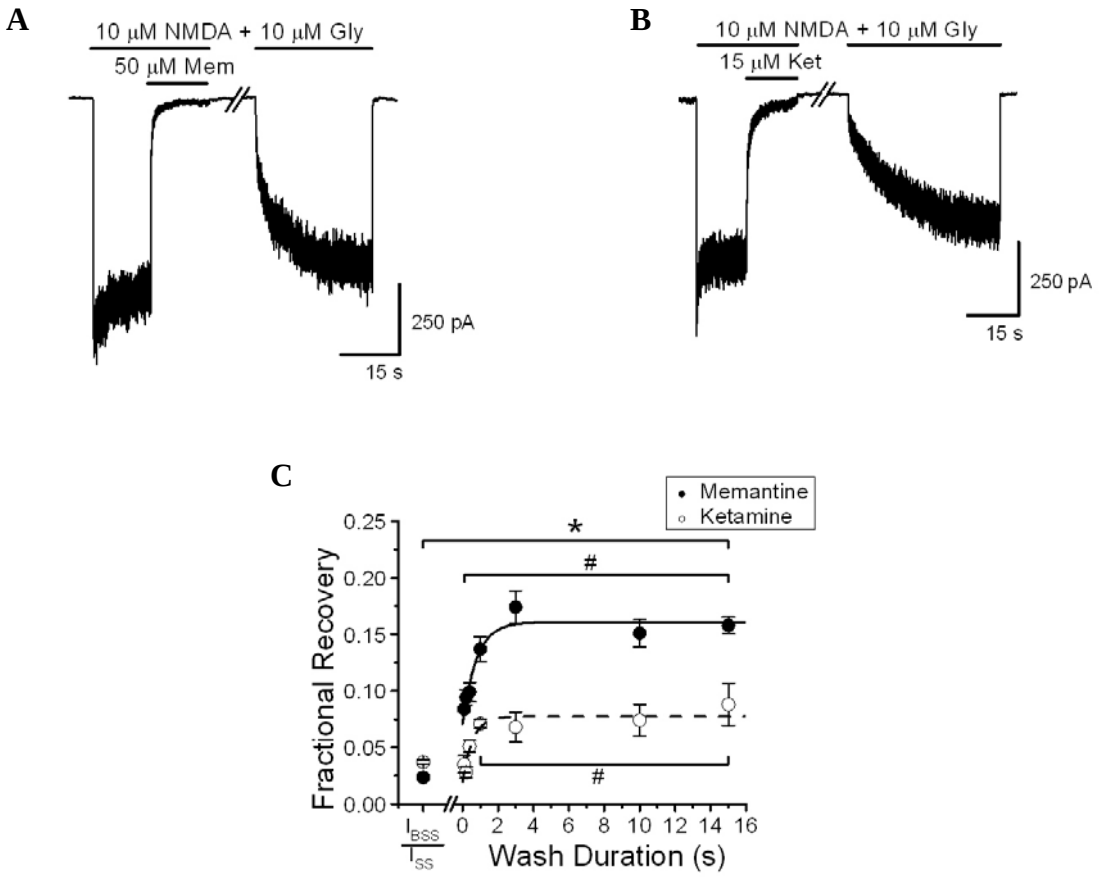

Figure 4. Time course of partial trapping development. A and B, NR1/2A receptor current traces during the double-pulse protocol that were used to measure the time course of recovery in the absence of agonist from inhibition by 50 μM memantine (A) or 15 μM ketamine (B). The break in the traces represents the wash duration which was varied (a wash duration of 15 s is shown in the sample traces). C, Plot of the time dependence of the fractional recovery from inhibition (see eqn (3)) by memantine (filled circles) or ketamine (open circles). Solid and dashed lines are single exponential fits (eqn (4)) to memantine and ketamine data, respectively. Residual responses (see eqn (1)) in the presence of memantine and ketamine are plotted as I_{BSS}/I_{SS}. The results are displayed as means ± SEM of 6 (memantine) or 4 (ketamine) cells. #

significantly ($p < .05$) different from respective I_{BSS}/I_{SS} (Bonferroni test). * significantly ($p < .05$) different between drugs (Student's t-test).

2.4.2 Partial trapping is not the result of open-channel escape

The unbinding rate of memantine from activated NMDA receptors increases as membrane voltage depolarizes (Parsons et al., 1993; Parsons et al., 1995; Parsons et al., 1996; Blanpied et al., 1997). We tested the contribution of the unbinding rate on the fractional recovery of memantine inhibition by applying a 5 s voltage jump to +54 mV (Figure 5A) during and after the time our previous data (Figure 4C) suggested partial trapping develops. If partial trapping of memantine results from escape through open channels, then the depolarizing voltage step should increase fractional recovery when applied shortly after removal of agonist and blocker from our cells. Figure 5B demonstrates no significant difference (ANOVA) of depolarizing voltage steps on the fractional recovery of memantine inhibition compared to controls, for which no voltage step was applied.

To further examine any contribution of unbinding rate to partial trapping, we followed an approach similar to that of Mealing et al. (2001), and modified the double-pulse protocol by continuing the application of memantine after the end of the agonist application (Figure 5C). Thus, a constant concentration of memantine was present throughout the time when non-trapped memantine dissociates (Figure 4C). We used this protocol to test the hypothesis that partial trapping is due to rapid memantine unbinding from the deep site before receptors close after removal of agonist and memantine. If this hypothesis is correct, then the continued presence of

memantine should maintain occupancy of the deep site, resulting in less fractional recovery. We found no significant change in the fractional recovery of memantine inhibition due to this extended application, compared to the double-pulse protocol in which memantine and agonist were removed simultaneously (Figure 5D). We feel that this result combined with the results of voltage jump experiments (Figure 5B) provide strong evidence that the relatively fast unbinding rate of memantine cannot fully explain its partial trapping by NR1/2A receptors.

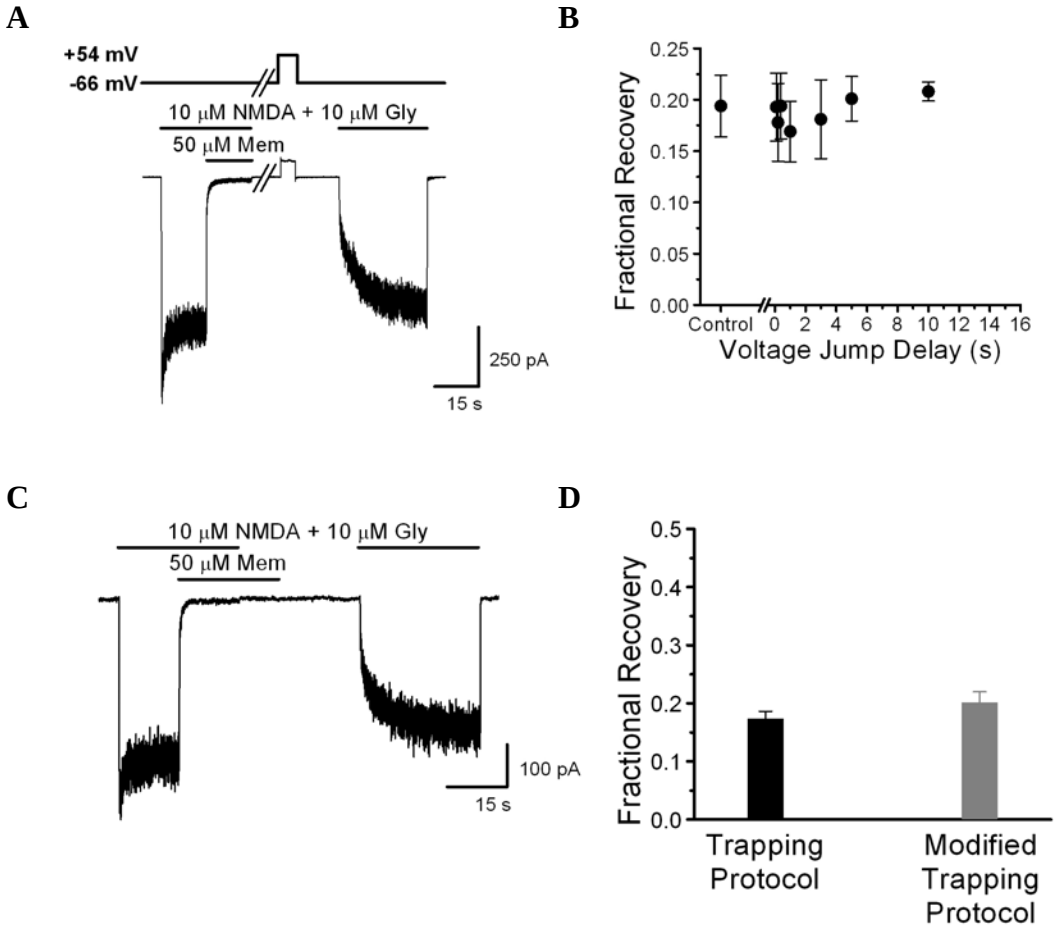

Figure 5. Non-trapped memantine does not escape from open channels. A, Voltage (upper) and current (lower) traces demonstrating the protocol used. Experiments were performed at -66 mV with 5 s depolarizing voltage steps to +54 mV beginning 0.1 – 10 s after removal of agonist and blocker. The break in the traces represents the time between agonist removal and agonist reapplication, which was varied (sample trace shows a 10 s wait before voltage step application). B, Membrane depolarizations applied during and after the development of partial trapping of 50 μM memantine had no significant effect (ANOVA) on fractional recovery (n = 5). Control data represent measurements made without a voltage step. C, Current trace demonstrating the modified trapping protocol. The modified trapping protocol was similar to the double-pulse protocol (Figure 2), except that blocker application was extended past the time when agonist application ended. Thus, blocker was present throughout the time when partial trapping developed (Figure 4C). D, Effects of the modified trapping protocol on fractional recovery of memantine inhibition. The continued presence of 50 μM memantine during the development of partial trapping (grey bar) had no significant effect (Student's t-test) on memantine trapping (n = 4). The normal "Trapping Protocol" data point (black bar) is replotted in Figure 8E (-66 mV memantine data).

2.4.3 NR1/2A receptor inhibition in the absence of agonist

Our finding that open-channel escape cannot fully account for the partial trapping of memantine led us to test a closed-channel mechanism. We explored the possibility that non-trapped memantine dissociates from a second inhibitory site from which unbinding can occur when the

channel is closed. When the channel is open there is competition between this site and the deep site for memantine binding (Figure 6). A previous study from this lab (Blanpied et al., 1997) demonstrated that memantine inhibits NMDA receptor currents at a superficial site accessible in the absence of agonist. We decided to test the hypothesis that memantine binding to and unbinding from this superficial site contributes to its partial trapping.

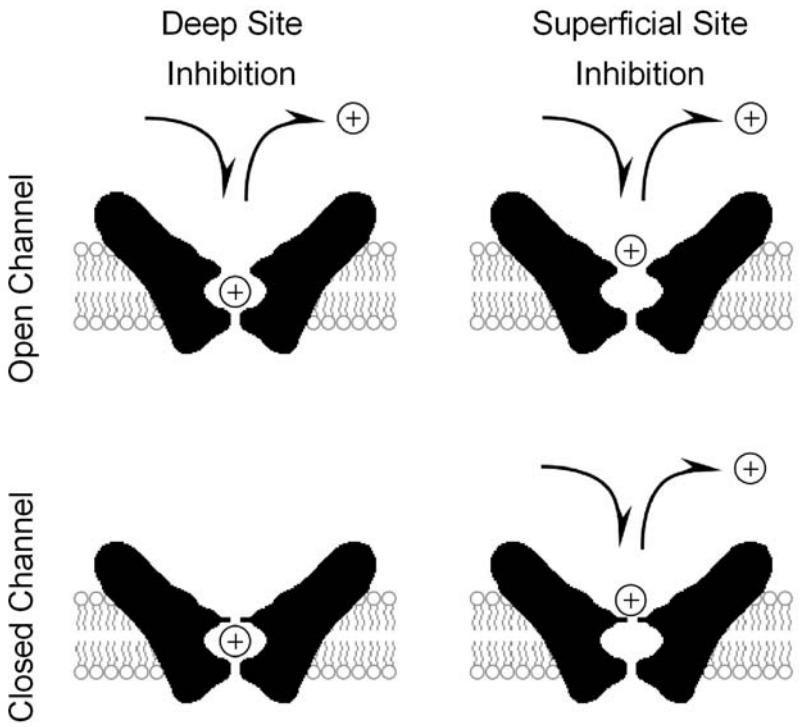

Figure 6. Competitive binding hypothesis of partial trapping of memantine. We hypothesized that partial trapping of memantine by NMDA receptors is the result of competitive binding at two inhibitory binding sites that cannot be occupied simultaneously. Blocker (represented by an open circle with +) can only be trapped when occupying the deep trapping site (bottom left). Blocker binding and unbinding at the deep site requires channel activation and is strongly

voltage-dependent. Memantine or ketamine can bind at this deep site (left side). Binding and unbinding of blocker at the superficial non-trapping site (right side) may occur whether or not channels are activated. Memantine but not ketamine (right side) can bind at the superficial site, which is located external to a trapping gate (broken line). Memantine unbinding from the superficial site after channel closure is hypothesized to result in partial trapping.

We measured a time course for memantine unbinding from the superficial site of NR1/2A receptors in the absence of agonist. If our hypothesis is correct, than we predict that this time course should be similar to the time course we measured for partial trapping development (Figure 4C). To measure the unbinding rate of memantine from the superficial site, we applied 500 μM memantine to our cells for 1 min in the absence of agonist, so that the deep site was inaccessible to blocker, varied the duration of a subsequent wash (0.4 – 15 s), and then reapplied agonist (Figure 7A, C). The high concentration of memantine was chosen because previous measurements (Blanpied et al., 1997) suggested that this site has a very low affinity for memantine. The peak current after agonist application revealed the extent of memantine inhibition resulting from occupancy at the superficial site. These experiments resulted in a time constant of $\tau = 1.94 \pm 0.19$ s for memantine unbinding from the superficial site (Figure 7C). There was a significant effect of wash duration on the recovery from occupancy at the superficial site by memantine ($p < 0.05$, ANOVA). *Post-hoc* tests showed a wash duration of either 3, 10, or 15 s resulted in significantly greater recovery from superficial site inhibition compared to the shortest wash duration, 0.4 s (p's < 0.05).

If partial trapping results from unbinding of memantine from the superficial non-trapping site, then the more fully trapped NMDA receptor channel blocker ketamine should exhibit much lower or no affinity at the superficial site. When we carried out the same experiment with ketamine (Figure 7B) we observed no significant (ANOVA) superficial site inhibition (Figure 7C), which suggests that ketamine inhibits NR1/2A receptors by binding only at the deep site. We measured significantly less recovery from memantine inhibition at the superficial site compared to the amount of recovery from ketamine at that site when wash durations of either 0.4, 1, or 3 s were applied (p's < 0.05).

To test how effectively memantine inhibits at the superficial site, we used the same protocol as shown in Figure 7A, and varied the concentration of memantine we applied in the absence of agonist. We measured, at the superficial site, an IC_{50} for memantine of 79.1 ± 20.2 μM at NR1/NR2A receptors at -66 mV, when a 0.4 s wash was applied between the application of memantine and the application of agonist (Figure 7D).

To be sure that our concentration-inhibition measurements represent steady-state inhibition at the superficial site, we varied the application for the lowest dose of memantine tested, 50 μM, in Figure 7D. The same protocol as shown in Figure 7A was used in these experiments, but the application of memantine in the absence of agonist was varied from 30 to 240 s. We found no significant effect of application duration (ANOVA) on the amount of inhibition at the superficial site by 50 μM memantine (Figure 7E), which suggests that the measurements presented in Figure 7D represent steady-state inhibition.

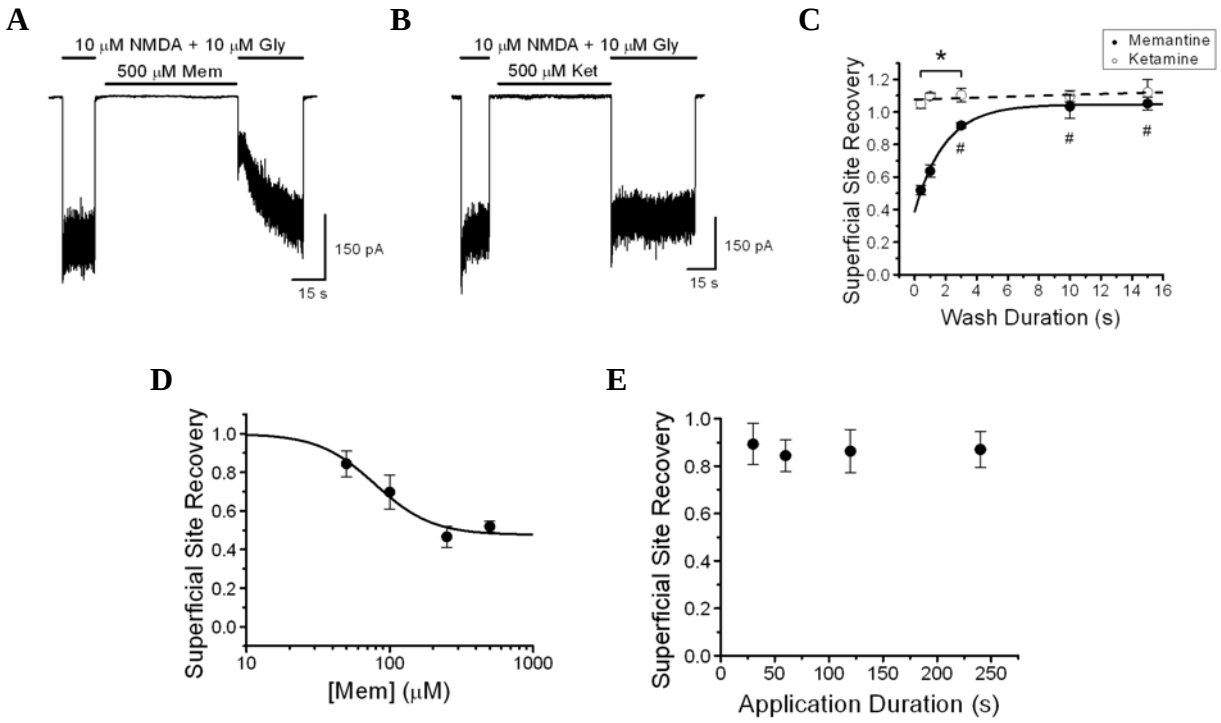

Figure 7. NMDA receptor inhibition by memantine but not ketamine at the superficial site. A and B, NR1/2A receptor current traces demonstrating the protocol used to measure memantine and ketamine inhibition in the absence of agonist. The wash duration following the application of 500 μM memantine (A) or 500 μM ketamine (B) and before the subsequent application of 10 μM NMDA was varied to measure the time constant of blocker unbinding from the superficial site. The traces presented are examples of a 0.4 s wash application. C, Measurements of the time course of recovery from inhibition at the superficial site by either 500 μM memantine (filled circles) or 500 μM ketamine (open circles) in the absence of agonist. Solid line is a single exponential fit (eqn (4)) to the data for 500 μM memantine (n = 4). Recovery from block by memantine at the superficial site was significantly affected by wash duration ($p < 0.05$, ANOVA) . Dashed line is a linear fit to the data for 500 μM ketamine (n = 4). There were no significant

differences (ANOVA) in recovery from block by ketamine between wash durations. D, Memantine concentration-inhibition curve for the superficial site. Measurements were gathered with the protocol demonstrated in A (wash duration of 0.4 s between memantine application and subsequent agonist application). Solid line represents the fit (eqn (6)) to the memantine data. E, The effect of the duration of memantine application in the absence of agonist on steady-state inhibition of NR1/NR2A receptors by 50 μM memantine (n = 3). The protocol was similar to the that shown in A, except the duration of memantine application was varied and the wash between memantine application and subsequent agonist application was held constant at 0.4 s. There were no significant differences (ANOVA) between the data points. # significantly ($p < 0.05$) different from respective 0.4 s wash (Bonferroni test). * significantly ($p < 0.05$) different between drugs (Student's t-test).

2.4.4 Voltage dependence of trapping

Blanpied et al. (1997) presented evidence that the superficial memantine binding site is located shallower within the voltage field of NMDA receptors than the deep site. This observation implies that memantine binding at the superficial site is less voltage dependent than the deep site. Thus, membrane voltages depolarized from rest should lead to greater superficial site binding, relative to binding at the deep site, and to less trapping. Bolshakov et al. (2003) demonstrated that the degree of trapping of a variety of NMDA receptor channel blockers is voltage dependent. We measured the fractional recovery from block by trapping of either memantine or ketamine in the channel of NR1/2A receptors voltage clamped at either -26, -66, and -106 mV (Figure 8A -

D). Memantine and ketamine concentrations were adjusted in these experiments to achieve a similar amount of inhibition at each voltage (Table 1), to prevent different amounts of blocker binding affecting our results. Our measurements demonstrate that the fractional recovery of inhibition by memantine is significantly dependent upon voltage ($p < 0.05$; ANOVA). *Post-hoc* tests showed the fractional recovery from inhibition by memantine at -106 mV tended to be less than that at -66 mV. Significantly more fractional recovery was observed at -26 mV than at -66 mV ($p < 0.05$, Figure 8 E). Consistent with our hypothesis, membrane voltage had less of an effect on the fractional recovery of ketamine inhibition (Figure 8C, D, E). Although there was a significant effect of membrane voltage on the fractional recovery of ketamine inhibition ($p < 0.05$; ANOVA), *post-hoc* testing showed that the amount of fractional recovery at neither -26 mV nor at -106 mV differed significantly from that measured at -66 mV. The fractional recovery of memantine inhibition at -66 mV and at -26 mV each were found to be significantly different from the fractional recovery of ketamine inhibition at the corresponding membrane voltages (p's < 0.05).

It is important to note that depolarization of the membrane voltage significantly affected the unbinding time constant of both memantine and ketamine from NR1/2A receptors (p's < 0.05, ANOVA, Table 1). *Post-hoc* tests showed that the unbinding time constant of memantine at -26 mV was significantly faster than that at -66 mV; and the unbinding time constant of ketamine at -106 mV was significantly slower than that at -66 mV ($p < 0.05$). Significant differences in the unbinding time constants between memantine and ketamine were found at all voltages tested (p's < 0.05). Although unbinding kinetics likely contributes to partial trapping, Figure 5B and D provide strong evidence that unbinding rate cannot be solely responsible.

Table 1. Residual current and unbinding time constant of memantine and ketamine at concentrations used to measure voltage dependence of trapping

Voltage (mV)	[Mem] (μM)	$I_{Mem}/I_{Control}$	Unbinding Time Constant (s)	[Ket] (μM)	$I_{Ket}/I_{Control}$	Unbinding Time Constant (s)
-26	180	0.020 ± 0.006	4.24 ± .42 # *	55	0.035 ± 0.002	11.2 ± 1.4 *
-66	50	0.020 ± 0.002	9.73 ± .49 *	15	0.044 ± 0.007	15.1 ± 1.4 *
-106	15	0.017 ± 0.007	8.76 ± 1.23 *	5	0.056 ± 0.003	25.8 ± 1.5 # *

Residual current values and unbinding time constants are presented as means ± SEM. # significantly ($p < 0.05$) different from respective value at -66 mV (Bonferroni test). * significantly ($p < 0.05$) different between drugs (Student's t-test).

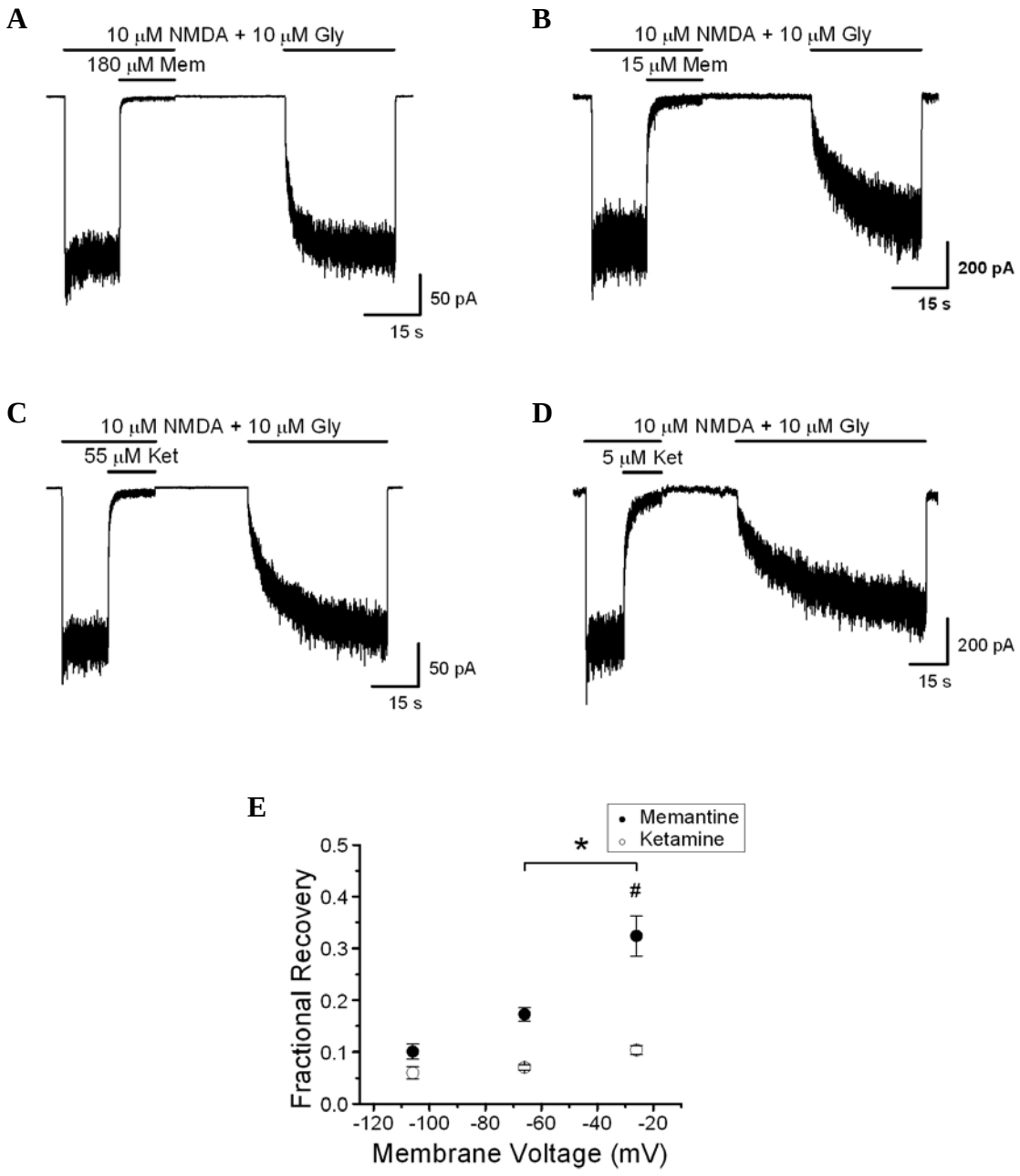

Figure 8. Effect of membrane voltage on fractional recovery of memantine and ketamine inhibition. A and B, Current traces recorded with the double-pulse protocol at -26 mV (A) and -106 mV (B) demonstrating the voltage dependence of memantine trapping. C and D, Current traces recorded at -26 mV (C) and -106 mV (D) demonstrating the voltage dependence of

ketamine trapping. E, Comparison of the effect of membrane voltage on fractional recovery from either memantine or ketamine inhibition. Inhibitor concentrations used at each voltage are listed in Table 1. Overall, the data show that fractional recovery of memantine inhibition is more strongly dependent on membrane voltage than recovery from ketamine inhibition. # memantine significantly ($p < 0.05$) different from respective value at -66 mV (Bonferroni test). * significantly ($p < 0.05$) different between drugs (Student's t-test).

2.4.5 Effects of $[ion]_o$ on memantine trapping

We tested the possibility that the superficial memantine binding site overlaps with the external cation binding site (Antonov et al., 1998; Antonov and Johnson, 1999; Zhu and Auerbach, 2001a, b). If the external cation binding site and the superficial memantine binding site overlap, then we may be able to alter memantine binding at the superficial site, relative to the deep site, by changing external ion concentration. We tested the effect of extracellular ion concentration ($[ion]_o$) on the degree of memantine trapping by decreasing the $[ion]_o$ of our solutions 4-fold (low $[ion]_o$ Ringers) .

We first measured the effect of a 4-fold reduction of $[ion]_o$ on the IC_{50} of memantine. The overall IC_{50} of memantine in the low $[ion]_o$ solution (0.86 ± 0.03 μM) was slightly lower than that in Normal Ringers solution (1.25 ± .04 μM; Figure 9A), consistent with the previous observation that lowering external cation concentration slightly lowers the IC_{50} of $Mg^{2+}{}_o$ (Qian et al., 2002). When we tested the effect of $[ion]_o$ on superficial site recovery of memantine inhibition we found a significant effect of wash duration, similar to what we found when using

Normal Ringers solution ($p < 0.05$, ANOVA, Figure 9B). *Post-hoc* tests revealed that the recovery from inhibition at the superficial site in low $[ion]_o$ was significantly greater after a wash duration of either 3, 10, or 15 s compared to recovery from inhibition after the shortest wash duration tested, 0.4 s. Significant differences in recovery from inhibition by memantine at the superficial site between Normal Ringers solution and $[low]_o$ solution were found at wash durations of either 0.4, 1, and 3 s (p's < 0.05).

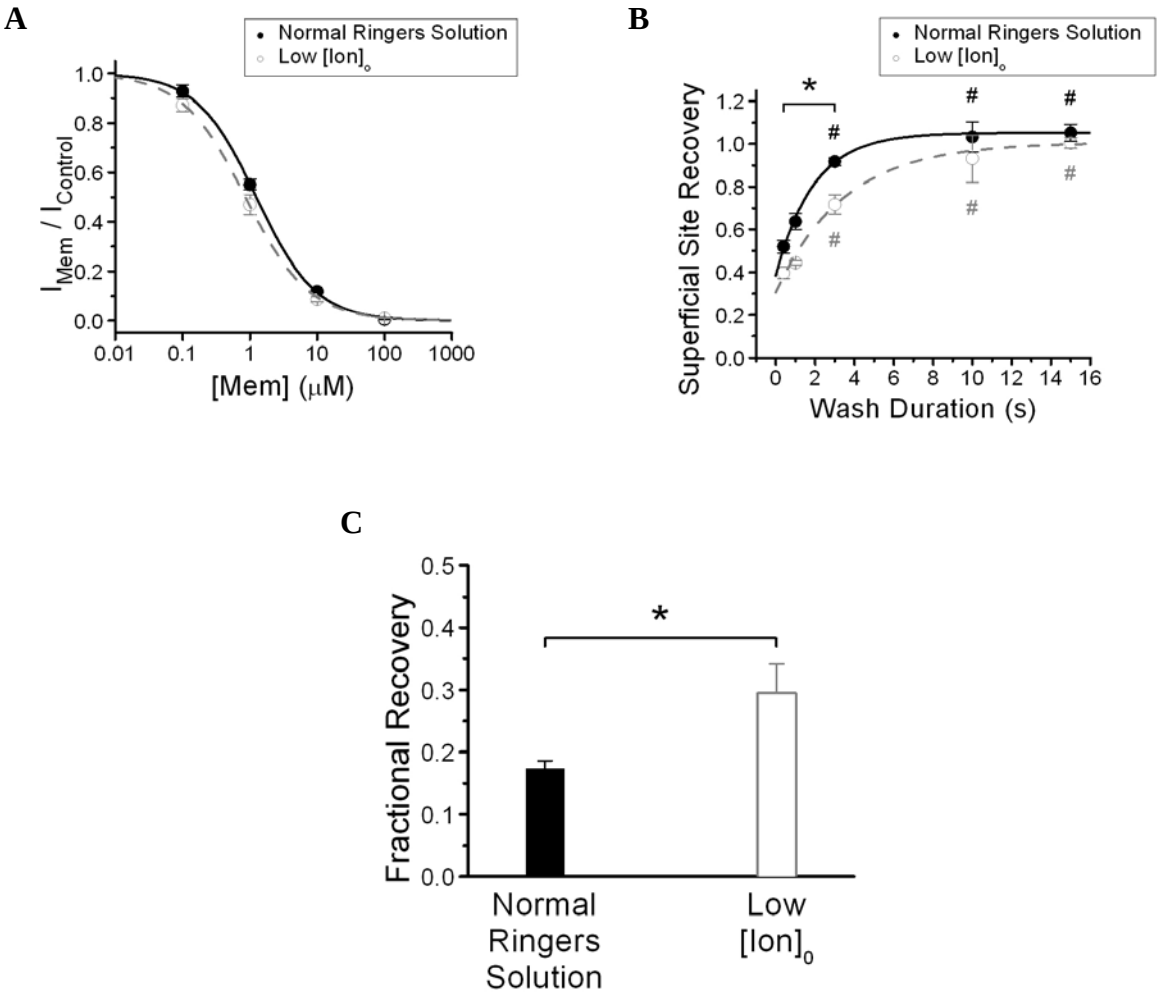

Figure 9. Effect of $[ion]_o$ on memantine trapping. A, Memantine concentration-inhibition curves recorded at -66 mV. Normal Ringers solution data (filled black circles, solid line) are replotted from Figure 3C. Decreasing $[ion]_o$ 4-fold (n = 4) slightly decreased the IC_{50} for memantine at NR1/2A receptors(open grey circles, dashed line) relative to the IC_{50} measured in control solution (filled black circles). B, Low $[ion]_o$ affects the unbinding of 500 μM memantine from the superficial site of NR1/NR2A receptors. Normal Ringers solution data (filled black circles, solid line) are replotted from Figure 7C (memantine data). Low $[ion]_o$ (n = 3) increased memantine binding to the superficial site (open grey circles, dashed line) and decreased unbinding rate (τ = 3.45 ± 0.52 s) compared to control solutions (τ = .1.94 ± 0.19 s). C, Low $[ion]_o$ significantly increased the fractional recovery of memantine inhibition. The Normal Ringers Solution (black bar) data are replotted from Figure 8E (-66 mV memantine data). Low $[ion]_o$ concentration (open grey bar) (n = 4) significantly decreased ($p < 0.05$, Student's t-test) trapping of memantine (50 μM). # significantly ($p < 0.05$) different from respective 0.4 s wash (Bonferroni test). * significantly ($p < 0.05$) different between extracellular solutions (Student's t-test).

Because decreasing $[ion]_o$ increased memantine binding at the superficial site, we should also observe less trapping of memantine by NR1/2A receptors if our two-site competition hypothesis is correct. Consistent with our hypothesis we observed significantly greater fractional recovery of memantine inhibition when we performed the double-pulse protocol in low $[ion]_o$ solution ($p < 0.05$, Figure 9C).

2.5 DISCUSSION

A lack of consensus on the mechanism of partial trapping (Blanpied et al., 1997; Chen and Lipton, 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Mealing et al., 1999; Mealing et al., 2001; Bolshakov et al., 2003), as well as inconsistencies between proposed hypotheses and experimental data, led us to explore further the mechanism by which memantine is partially trapped by NR1/2A NMDA receptors. Memantine binding to two separate sites on NMDA receptors was demonstrated previously (Blanpied et al., 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Chen and Lipton, 2005), a property that was found not to apply to the full trapping NMDA receptor channel blocker ketamine. We tested the hypothesis that the partial trapping of memantine is the result of competitive binding at two sites on NR1/2A receptors, that cannot be occupied simultaneously; a deep trapping site and a superficial non-trapping site that is accessible regardless of whether the channel is open or closed (Figure 6).

2.5.1 Partial-trapping development

We determined that non-trapped memantine dissociates from NR1/2A receptors over a time course of $\tau = 0.78 \pm 0.29$ s (Figure 4C). The fractional recovery of memantine inhibition was measured to be ~17 %, which is consistent with measurements by others (Blanpied et al., 1997). We also found evidence that, during the time partial trapping develops, non-trapped memantine does not escape from an open channel (Figure 5B, D). The fractional recovery of ketamine inhibition we measured was significantly less than that of memantine inhibition at all of the wash durations tested (Figure 4C). The small amount of fractional recovery we measured with the

full-trapping blocker, ketamine, may have arisen from an effect of ketamine on NMDA receptor desensitization, which we attempted to but could not fully control for.

2.5.2 Memantine inhibition of NR1/2A receptors in the absence of agonist

If the competitive binding hypothesis is correct, unbinding from the superficial site should follow a time course similar to that measured for the development of partial trapping. The previous estimate of the unbinding time constant of memantine from the superficial site of NMDA receptors was very similar to the time constant measured for unbinding from the deep site (Blanpied et al., 1997), and much slower than what we measured for partial trapping development (Figure 4C). The protocol previously used to measure unbinding from the superficial site may have allowed memantine to reach the deep site. Repeated brief agonist applications were delivered in short intervals 1 s after the washout of memantine from the cell. It is possible that memantine bound at the superficial site during the first agonist application after memantine washout could have been pulled through the membrane voltage field towards the deep site and trapped upon agonist removal. Subsequent agonist applications in the absence of memantine then would have measured memantine escape from the deep site, leading to the similar unbinding time constants reported for both sites. Our measurements of inhibition at the superficial site were based on the first application of agonist after blocker exposure (Figure 7A, B), which should have prevented deep site binding. The unbinding time constant of memantine we measured at -66 mV for unbinding from the superficial site ($\tau = 1.94 \pm 0.19$ s, Figure 7C) is much faster than the time constant for unbinding from the deep site ($\tau = 9.73 \pm 0.49$ s, Table 1), and closer to that measured for the development of partial trapping ($\tau = 0.78 \pm 0.29$ s). Although the time constants we measured for the development of partial trapping and memantine

unbinding from the superficial site are not the same, there are inaccuracies inherent in the indirect way our measurements were made. Thus, it is plausible that the time course of partial trapping development does reflect memantine unbinding from the superficial site. Ketamine did not inhibit NR1/2A-mediated responses at the superficial site (Figure 7C), which suggests that ketamine inhibition of NMDA receptors can occur only at the deep trapping site.

The memantine IC_{50} we measured for the superficial site (79.1 ± 20.2 μM, Figure 7D) is lower than the IC_{50} reported previously (Blanpied et al., 1997). Possible explanations for the differences between our measurements and those of Blanpied et al. (1997) include receptor subunit composition and curve fitting of the data. Blanpied et al. measured a memantine superficial site IC_{50} at NMDA receptors expressed in cultured cortical neuron that express NR1, NR2A, and NR2B subunits and reported an IC_{50} value of 179 μM, at -67 mV. The data presented here were gathered for NR1/2A receptors transiently transfected into HEK293T cells. It is unclear how the presence of NR2B subunits, as well cell type, might affect memantine IC_{50} for the superficial site.

Furthermore, our concentration-inhibition data was best fit with maximal inhibition by memantine at the superficial site being ~50 % (Figure 7D). Blanpied et al. (1997) fit their data with an equation that assumed 100 % inhibition at high memantine concentration. Since we waited 0.4 s after blocker washout before agonist was applied, allowing some memantine unbinding, maximum inhibition could not be 100 %. Therefore, our data were fit with a curve that allowed maximum inhibition at the superficial site to be adjustable.

We used a kinetic model of NMDA receptors to determine whether competitive binding of memantine at a superficial site and the deep trapping site would result in partial trapping (data not shown). Model simulations reproduced partial trapping, but with a trapping site IC_{50} that we

measured (1.25 μM), a superficial site IC_{50} of ~30 μM was required to recreate the degree of partial trapping we observed experimentally (~17%). While this superficial site IC_{50} value is lower than our experimentally measured value (79.1 μM), it is possible that our indirect protocol for measuring inhibition at the superficial site overestimated the IC_{50}. In addition, we were able to estimate only the IC_{50} of memantine for the superficial site based on binding that occurred when NMDA receptor channels were closed. It is possible that the IC_{50} of memantine for the superficial site is lower when channels are open.

2.5.3 Voltage dependence of trapping

We attempted to vary the amount of memantine binding at the deep site, relative to the superficial site, by running the trapping protocol at various membrane voltages. We hypothesized that depolarizing the membrane voltage would cause a greater increase in the IC_{50} of memantine for the deep site than the IC_{50} for the superficial site, leading to greater memantine binding at the superficial site and less trapping. Alternatively, hyperpolarizing the membrane voltage should favor deep site binding and greater memantine trapping.

Memantine and ketamine trapping was significantly affected by membrane voltage. However, ketamine was more fully trapped than memantine at all voltages tested. Ketamine's lack of affinity for the superficial site would explain the lesser effect of membrane voltage on its trapping; however, the significant effect of voltage we measured may reflect a contribution of faster ketamine unbinding at more depolarized voltages (Table 1). The unbinding time constants of memantine and ketamine were also significantly affected by membrane voltage. Therefore, although these data are in agreement with our hypothesis, they leave open the possibility that

membrane voltage may have affected trapping because of affects on unbinding kinetics rather than competition between the superficial and deep sites.

2.5.4 Superficial memantine binding site may overlap the external cation binding site of NR1/2A receptors

We tested whether the superficial memantine binding site on NR1/2A receptors overlaps the binding site for external cations. We lowered $[ion]_o$ 4-fold and tested whether or not memantine binding at the superficial site, as well as trapping, was affected. Low $[ion]_o$ slightly lowered the deep site IC_{50} of memantine compared to the IC_{50} value measured in control $[ion]_o$ (Figure 9A). We found that lowering $[ion]_o$ significantly increased memantine binding at the superficial site, compared binding at that site in control solution, after wash durations of either 0.4, 1, and 3 s (Figure 9B). Significant differences were not measured at longer wash durations because recovery from block was nearly complete in both conditions. Consistent with our hypothesis, the decrease in binding of memantine at the superficial site, compared to binding at the deep site in low $[ion]_o$ was accompanied by a significant increase in fractional recovery (Figure 9C). These data provide further support for a role of the superficial memantine binding site in partial trapping.

Our finding that $[ion]_o$ affects memantine binding at the superficial site suggests there may be overlap between the binding site for extracellular cations and the superficial memantine binding site. A region of the NR1 subunit located just after the C-terminal end of the M3 transmembrane region has been demonstrated through mutational analysis to modulate Ca^{2+} influx through NMDA receptors (Watanabe et al., 2002). A string of amino acids (DRPEER) with a net negative charge forms a region that binds Ca^{2+} through an electrostatic interaction. It

is possible that the DRPEER site could contribute to the superficial site binding of positively charged memantine. However, the DRPEER site is located outside of the voltage field of NMDA receptors (Sobolevsky et al., 2002). Memantine binding to the superficial site is voltage-dependent (Blanpied et al., 1997), which argues against the suggestion that the DRPEER site contributes to the superficial site. However, it is possible that the DRPEER site is only part of the superficial memantine binding site and that other regions, located within the voltage field, also contribute this site.

3.0 MG^{2+} IMPARTS NMDA RECEPTOR SUBTYPE SELECTIVITY TO THE ALZHEIMER'S DRUG MEMANTINE

3.1 ABSTRACT

Research on memantine's mechanism of action has focused on the NMDA receptor subtypes most highly expressed in adult cerebral cortex, NR1/2A and NR1/2B receptors (Cull-Candy and Leszkiewicz, 2004), and has largely ignored interactions with extracellular Mg^{2+} (Mg^{2+}_o). Mg^{2+}_o is an endogenous NMDA receptor channel blocker that binds near memantine's binding site (Kashiwagi et al., 2002; Chen and Lipton, 2005). We report that a physiological concentration (1 mM) of Mg^{2+}_o at resting membrane potential decreased memantine inhibition of NR1/2A and NR1/2B receptors nearly 20-fold, but decreased memantine inhibition of the other principal NMDA receptor subtypes, NR1/2C and NR1/2D receptors, only ~3-fold. As a result, therapeutic memantine concentrations should have negligible effects on NR1/2A or NR1/2B receptor activity but pronounced effects on NR1/2C and NR1/2D receptors. We report similar results with the NMDA receptor channel blocker ketamine, a drug used to model schizophrenia (Krystal et al., 2003). These results suggest that currently hypothesized mechanisms of memantine and ketamine action should be reconsidered. NR1/2C and/or NR1/2D receptors play a more important role in cortical physiology and pathology than previously appreciated.

3.2 INTRODUCTION

Functional NMDA receptors are composed of 4 subunits, and usually contain NR1 and one or more of the four NR2 (NR2A – NR2D) subunits (Dingledine et al., 1999). NR2 subunit expression is developmentally and regionally regulated (Monyer et al., 1994; Cull-Candy and Leszkiewicz, 2004). The identity of the NR2 subunit(s) in a NMDA receptor strongly influences receptor properties, including agonist and competitive antagonist affinities, deactivation kinetics, single-channel conductance, calcium (Ca^{2+}) permeability, and characteristics of Mg^{2+}_o channel block (Dingledine et al., 1999; Cull-Candy and Leszkiewicz, 2004). The high Ca^{2+} permeability of NMDA receptors and regulation of Ca^{2+} influx by voltage-dependent channel block by Mg^{2+}_o are properties of fundamental physiological and pathological importance. Of particular relevance to the results presented here, NMDA receptor inhibition by Mg^{2+}_o is considerably weaker in NR1/2C and NR1/2D receptors than in NR1/2A or NR1/2B receptors (Kutsuwada et al., 1992; Monyer et al., 1994).

Several drugs of clinical importance, including memantine, act by binding in the channel of NMDA receptors at a site that overlaps with the Mg^{2+}_o blocking site (Kashiwagi et al., 2002; Chen and Lipton, 2005). Like Mg^{2+}_o, memantine block exhibits voltage dependence; the concentration of memantine that inhibits NMDA responses by 50% (the IC_{50}) increases with depolarization (Rogawski and Wenk, 2003; Johnson and Kotermanski, 2006; Parsons et al., 2007). Memantine alleviates the cognitive decline associated with Alzheimer's disease, and represents a major departure in Alzheimer's disease therapy from previously-developed medications that enhance cholinergic transmission (Schmitt, 2005; Lipton, 2006). Extensive evidence indicates that the therapeutic effects of memantine derive predominantly from NMDA

receptor inhibition (Rogawski and Wenk, 2003; Schmitt, 2005; Lipton, 2006; Parsons et al., 2007).

3.3 MATERIALS AND METHODS

3.3.1 Cell culture and transfection

Experiments were performed on the HEK293T mammalian cell line. Cells were transfected using Lipofectamine (Invitrogen Corporation, Carlsbad, CA), as previously described (Qian and Johnson, 2006) with cDNA for the NR1-1a (GenBank accession number (ACCN) X63255, in pcDM8) subunit and either the NR2A (ACCN M91561, in pcDM8), NR2B (ACCN M91562, in pcDNA1), NR2C (ACCN M91563, in pcDNA1), or NR2D (ACCN L31612, in pcDM8) subunit. cDNA for enhanced Green Fluorescent Protein (eGFP) was co-transfected as a marker of successful transfection.

3.3.2 Electrophysiology

Whole-cell patch-clamp recordings were performed with an Axopatch-1D amplifier ~24 hours after transfection. For experiments performed in 1 mM Mg^{2+}_o, cells with bright eGFP fluorescence (presumably associated with larger NMDA responses) were chosen. Recording electrodes of 2 to 6 MΩ resistance were filled with an internal solution consisting of (in mM): 125 CsCl, 10 BAPTA, and 10 HEPES, with pH adjusted to 7.2 ± 0.05 with CsOH and osmolality of 275 ± 10 mmol/kg. Series resistance was compensated 80 to 90%.

External solutions consisted of (in mM): 140 NaCl, 2.8 KCl, 1 $CaCl_2$, and 10 HEPES (and 1 $MgCl_2$ when indicated), with pH adjusted to 7.2 ± 0.05 with NaOH and osmolality adjusted to 290 ± 10 mmol/kg with sucrose. Solutions were applied to cells by a seven-barrel, gravity-fed fast perfusion system. Concentration-inhibition data were measured in the continuous presence of either 0 or 1 mM Mg^{2+}_o during application of 1 mM glutamate and 100 μM glycine ("agonists") with serial application of 5 concentrations of either memantine or ketamine ranging from 0 to up to 1 mM (see Figures 10 and 11). A subsequent agonist application always was made, and data were rejected if the steady-state response to agonists following blocker washout did not recover to at least 70% of the response to agonists that preceded blocker application. Correction for the measured junction potential of -6 mV was applied to all data.

3.3.3 Data analysis

Data were low-pass filtered at 100 Hz and analyzed using Clampfit 9.2 (Axon Instruments, Union City, CA). Current averages over a 500 msec time window were used to obtain values of baseline current and steady-state NMDA receptor-mediated current, without or with memantine, ketamine, and/or Mg^{2+}_o as indicated. Concentration-inhibition curves were fit using the equation

$$I_{Mem}/I_{Con} \text{ or } I_{Ket}/I_{Con} = 1/(1 + ([B] /IC_{50})^{n_H})$$

where I_{Mem} and I_{Ket} are steady state currents in agonists + blocker(s), I_{Con} is steady-state current in agonists alone, [B] is the concentration of memantine or ketamine, and n_H is the Hill coefficient.

3.4 RESULTS

Identification of an inhibitory drug's site(s) of action requires knowledge of its IC_{50} at potential targets under physiological conditions. The median value of the many published measurements of memantine's IC_{50} for NMDA receptors at voltages near rest is ~1 μM (Johnson and Kotermanski, 2006; Parsons et al., 2007). Memantine has been found to exhibit only weak NMDA receptor subtype selectivity, with an IC_{50} for NR1/2A receptors ~2 to several-fold higher than for NR1/2B, NR1/2C, and NR1/2D receptors (Dravid et al., 2007; Parsons et al., 2007). Use of these data to evaluate memantine's potential interactions with NMDA receptors under physiological conditions suffers from a potentially critical oversight: endogenous Mg^{2+} may compete with or otherwise affect memantine binding. Previous estimates of memantine affinity, selectivity, and voltage dependence have been performed in the absence of Mg^{2+}_o, possibly because of difficulty in measuring memantine inhibition of the small NMDA responses observed in physiological Mg^{2+}_o. However, evidence for overlap of memantine and Mg^{2+}_o binding sites (Kashiwagi et al., 2002; Chen and Lipton, 2005) and hindrance of memantine binding by Mg^{2+}_o (Sobolevsky et al., 1998) suggest that physiological Mg^{2+}_o could powerfully influence memantine inhibition of NMDA receptors. If Mg^{2+}_o and memantine compete for binding in the channel, then the IC_{50} of memantine should increase in the presence of Mg^{2+}_o. We therefore examined memantine inhibition of NMDA receptors in the presence of a physiological (1 mM) concentration of Mg^{2+}_o.

We compared memantine inhibition of whole-cell currents recorded at −66 mV from HEK293T cells transfected with cDNAs encoding the NR1 and either the NR2A, NR2B, NR2C, or NR2D subunits in 0 and 1 mM Mg^{2+}_o. NMDA responses were activated with saturating concentrations of glutamate (1 mM) and glycine (100 μM) to avoid possible agonist

concentration dependence of memantine inhibition (Lipton, 2006) (but see Parsons et al., 2007). Results of experiments in 0 Mg^{2+}_o demonstrated that memantine exhibits little NMDA receptor subtype selectivity, consistent with previous measurements. Memantine IC_{50}s for all NMDA receptor subtypes were between 0.5 and 1 μM (Figure10A, B, Table 2), with the IC_{50} for NR1/2A receptors slightly higher than for other receptor subtypes.

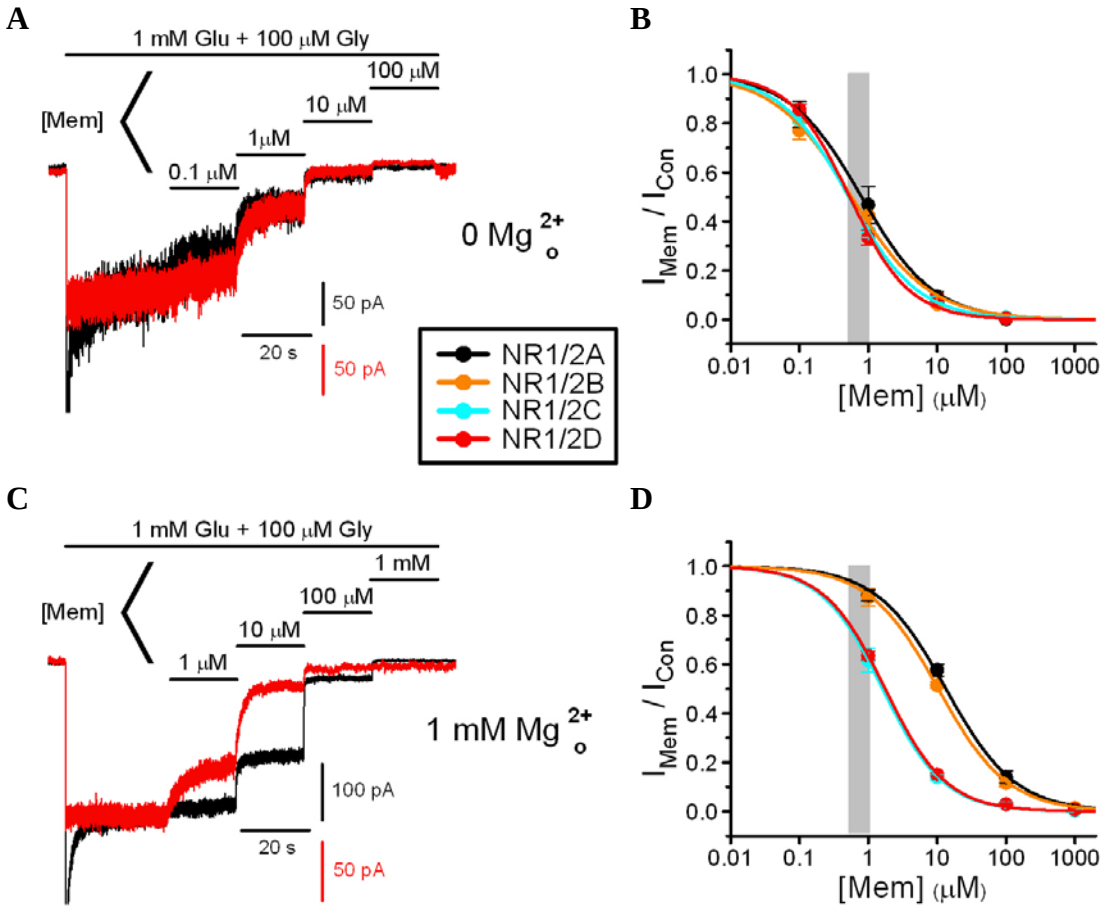

Figure 10. Effects of Mg^{2+}_o and NMDA receptor subunit composition on memantine inhibition. A and B, Overlay of NR1/2A (black) and NR1/2D (red) receptor current traces recorded from

transfected HEK293T cells in 0 Mg^{2+}_o (A) and 1 mM Mg^{2+}_o (C) during application of the indicated concentrations of memantine. Peak inward NR1/2A receptor currents here and in Figure 11 are truncated. Similar experiments were performed with NR1/2B and NR1/2C receptors (examples not shown). B and D, Memantine concentration-inhibition curves recorded in 0 Mg^{2+}_o (B) and 1 mM Mg^{2+}_o (D) from HEK293T cells transfected with the indicated NMDA receptor subtype. Memantine shows almost no subunit selectivity in 0 Mg^{2+}_o (B) but is selective for NR1/2C and NR1/2D receptors in 1 mM Mg^{2+}_o (D). Grey shaded areas represent estimated free extracellular memantine concentration range (0.5 – 1 μM) during typical therapeutic treatment (Parsons et al., 2007).

Under conditions similar to those used here, 1 mM Mg^{2+}_o inhibits responses of NR1/2A and NR1/2B receptors by ~95%, and responses of NR1/2C and NR1/2D receptors by ~80% (Clarke & Johnson, unpublished observations and Monyer et al., 1994). Use of transfected HEK293T cells with large NMDA receptor-mediated currents nevertheless permitted accurate measurement of memantine concentration-inhibition curves in 1 mM Mg^{2+}_o. The memantine IC_{50} was influenced strongly, and in a subtype-selective manner, by 1 mM Mg^{2+}_o (Figure 10C, D, Table 2): memantine concentration-inhibition curves were right-shifted by factors of 16.8 (NR1/2A), 18.2 (NR1/2B), 3.1 (NR1/2C), and 3.3 (NR1/2D). As a result, in 1 mM Mg^{2+}_o, memantine acquires a 5.9- to 8.3-fold selectivity for NR1/2C and NR1/2D receptors over NR1/2A and NR1/2B receptors.

We also investigated the effects of Mg^{2+}_o on NMDA receptor inhibition by ketamine, another channel blocker of broad clinical and pathological significance that exhibits kinetics and

IC_{50}s similar to memantine's. Ketamine is an important tool in schizophrenia research because it is psychotomimetic in healthy adults, exacerbates symptoms in schizophrenics (Krystal et al., 2003), and is commonly used to generate animal models of schizophrenia (Moghaddam and Jackson, 2003). Despite its psychotomimetic properties in adult humans, ketamine is used as a general anesthetic in children and animals, and demonstrates potential in the treatment of other conditions, including neuropathic pain (Annetta et al., 2005). Ketamine has been reported to be largely unselective among NMDA receptor subtypes (Yamakura et al., 1993; Dravid et al., 2007). However, as with memantine, nearly all studies of the IC_{50} of ketamine and subtype selectivity have been performed in 0 Mg^{2+}_o; when interactions have been examined, NMDA receptor inhibition by ketamine has been reported both to be both reduced (MacDonald et al., 1991) and augmented (Liu et al., 2001) by Mg^{2+}_o.

Our ketamine IC_{50} measurements in 0 and 1 mM Mg^{2+}_o are shown in Figure 11 and Table 2. The effects of 1 mM Mg^{2+}_o on ketamine inhibition strongly resembled the effects on memantine inhibition: ketamine concentration-inhibition curves were right-shifted by factors of 16.2 (NR1/2A), 16.4 (NR1/2B), 2.3 (NR1/2C), and 3.6 (NR1/2D). Thus, the powerful effects of Mg^{2+}_o are not specific to memantine, a conclusion also supported by previous data indicating that the ketamine analog phencyclidine interacts competitively with Mg^{2+}_o when inhibiting NMDA responses (Lerma et al., 1991). Because of the structural dissimilarity of memantine and ketamine, the results presented here suggest that NMDA receptor channel-blocking drugs in general interact competitively with Mg^{2+}_o.

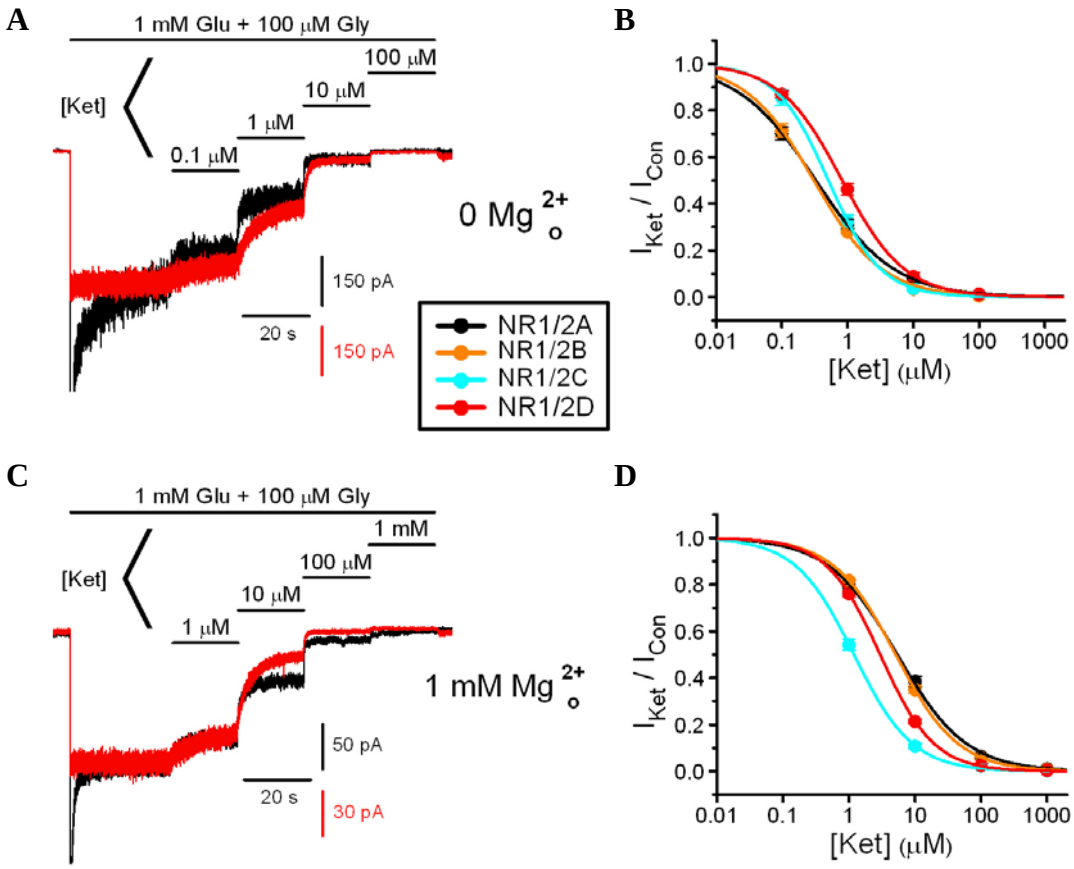

Figure 11. Effects of Mg^{2+}_o and NMDA receptor subunit composition on ketamine inhibition. A and C, Overlay of NR1/2A (black) and NR1/2D (red) receptor current traces recorded from transfected HEK293T cells in 0 Mg^{2+}_o (A) and in 1 mM Mg^{2+}_o (C) during application of the indicated concentrations of (+/−) ketamine. Similar experiments were performed with NR1/2B and NR1/2C receptors (examples not shown). B and D, Ketamine concentration-inhibition curves recorded in 0 Mg^{2+}_o (B) and in 1 mM Mg^{2+}_o (D) from HEK293T cells transfected with the indicated NMDA receptor subtype.

Table 2. Effect of Mg^{2+}_o on IC_{50}s for memantine and ketamine inhibition of NMDA receptors.

		NMDA Receptor Subtype						
		NR1/2A	NR1/2B		NR1/2C		NR1/2D	
Drug	$[Mg^{2+}]_o$ (mM)	IC_{50}	IC_{50}	Ratio $\frac{2A}{2B}$	IC_{50}	Ratio $\frac{2A}{2C}$	IC_{50}	Ratio $\frac{2A}{2D}$
Memantine	0	0.80 ± 0.07	0.57 ± 0.09	1.40	0.52 ± 0.02	1.54	0.54 ± 0.06	1.48
	1	13.4 ± 1.3	10.4 ± 0.4	1.29	1.61 ± 0.06	8.32	1.76 ± 0.06	7.61
Ketamine	0	0.33 ± 0.01	0.31 ± 0.02	1.06	0.51 ± 0.01	0.65	0.83 ± 0.02	0.40
	1	5.35 ± 0.34	5.08 ± 0.02	1.05	1.18 ± 0.04	4.53	2.95 ± 0.02	1.81

All IC_{50} values are in μM. IC_{50} values measured using concentration-inhibition curves are given as mean ± SEM, and are based on measurements from 3-4 cells for each condition. Each ratio is the IC_{50} for NR1/2A receptors divided by the IC_{50} for the indicated NMDA receptor subtype.

3.5 DISCUSSION

The above results show a powerful effect of Mg^{2+}_o on the IC_{50}, selectivity of clinically relevant NMDA receptor channel blockers. The IC_{50} increases take place in a critical concentration range. The free extracellular memantine concentration in the brain of patients following typical

treatment regimes is estimated to be ~0.5 – 1 μM (Parsons et al., 2007). The similarity of the therapeutic concentration of memantine to its IC_{50} for NMDA receptors measured in 0 Mg^{2+}_o has been critical in identifying NMDA receptors as the site of memantine action. Because of memantine's weak NMDA receptor subtype selectivity in 0 Mg^{2+}_o (Dravid et al., 2007; Parsons et al., 2007) and the high expression of NR2A and NR2B subunits in cortex (Cull-Candy and Leszkiewicz, 2004), research generally has focused on memantine interactions with NR2A and/or NR2B-containing receptors (Blanpied et al., 1997; Kashiwagi et al., 2002; Chen and Lipton, 2005; Parsons et al., 2007). It has also been suggested that the therapeutic utility of memantine may be related to its weak selectivity for NR2C and NR2D containing receptors observed in 0 Mg^{2+}_o (Rogawski and Wenk, 2003; David et al., 2006). Our data show that in physiological Mg^{2+}_o and at voltages near rest, the memantine IC_{50} for NR1/2A and NR1/2B receptors is over an order of magnitude higher than therapeutic brain concentrations (Figure 10, Table 2). 1 mM Mg^{2+}_o has a far weaker effect on the IC_{50} of memantine (as well as ketamine, and probably other channel blockers) for NR1/2C and NR1/2D receptors, for two reasons: first, because Mg^{2+}_o inhibits NR1/2C and NR1/2D receptors with relatively high IC_{50}s; and second, because of incomplete competition between Mg^{2+}_o and memantine (or ketamine) at NR1/2C and NR1/2D receptors. These data suggest that NR2C and/or NR2D containing NMDA receptors are likely sites of pharmacological memantine action. Memantine also inhibits other receptors with low IC_{50}s, including nicotinic acetylcholine receptors (nAChRs) (Aracava et al., 2005; Parsons et al., 2007). Inhibition of nAChRs appears unlikely to be the principal basis of memantine's therapeutic utility in Alzheimer's disease (Johnson and Kotermanski, 2006; Parsons et al., 2007), since Alzheimer's disease treatments other than memantine are aimed at augmenting cholinergic transmission.

The clinical effects of memantine and ketamine suggest that preferential inhibition of NR2C and/or NR2D containing NMDA receptors can strongly impact cognitive function. NR2C and NR2D subunits are expressed in the adult mammalian hippocampus and cortex, brain regions extensively involved in Alzheimer's disease and schizophrenia, although at lower levels than NR2A or NR2B subunits. Pathological activation, by long exposure to low levels of glutamate, of extrasynaptic NR2D containing receptors could raise the overall synaptic noise, making detection of physiologically relevant signals difficult (Parsons et al., 2007). A possible consequence of inhibition of NR2D containing NMDA receptors is preferential reduction of tonic NMDA receptor-mediated pyramidal cell currents, thereby decreasing synaptic noise (Le Meur et al., 2007). Alternatively, inhibition of NR2D containing receptors could selectively reduce excitation of the subset of inhibitory neurons that highly express NR2D (Monyer et al., 1994), resulting in disinhibition of excitatory neurons, thereby enhancing physiologically relevant signals. In Alzheimer's disease, amyloid-β accumulation (especially in excitatory pyramidal neurons (D'Andrea and Nagele, 2006) can cause internalization of NMDA receptors (Snyder et al., 2005) and preferential loss in cortex of excitatory terminals (Bell and Claudio Cuello, 2006). Memantine could partially counterbalance these effects by preferentially inhibiting interneuronal NMDA receptors; if this suggestion is correct, then more selective inhibition of NR1/2D receptors may hold therapeutic promise. The clinical utility of memantine may be enhanced by its inhibition of α-7 nAChRs (Aracava et al., 2005), which participate in amyloid-β-induced NMDA receptor internalization (Snyder et al., 2005). Ketamine shows a similar (although weaker) preferential inhibition of NR1/2C and NR1/2D receptors, which suggests more complex subtype selectivity. Nevertheless, ketamine's selective action at NR2C containing receptors at normal resting potential also may lead to cortical disinhibition, a process

hypothesized to be responsible for ketamine's ability to induce a schizophrenia-like psychotic state (Greene, 2001). These ideas emphasize the importance of understanding the roles played by NR2C and NR2D subunits in cortical function, and the mechanisms that underlie the diverse clinical actions of NMDA receptor channel blockers.

4.0 DIRECT COMPARISON OF THE LOCOMOTOR AND COGNITIVE EFFECTS OF THE NMDA RECEPTOR ANTAGONISTS MEMANTINE AND KETAMINE IN RATS

4.1 ABSTRACT

Conflicting results have been reported on the extent of behavioral effects elicited by some NMDA receptor antagonists in rats, which may be attributable in part to differences in drug administration protocols, as well as animal strain, age, and sex. Many studies tested the antagonist's effects at only one time delay after administration of the drug, ignoring the pharmacokinetic differences that exist among many of the drugs tested. We directly compared, in adult male Sprague-Dawley rats, the effects of two drugs that inhibit NMDA receptor activity with similar affinity and kinetics, but very different pharmacokinetic profiles. Intraperitoneal injections of either 0, 5, 10, 20, or 40 mg/kg doses of either memantine or of ketamine were administered and effects on behavior in an activity monitor and in a spontaneous alternation task were measured. To examine how pharmacokinetic differences between these two drugs affect behavior, animals were tested either 15 or 45 min after drug administration. Both memantine and ketamine decreased ambulatory activity and rearings when the context of the testing environment was novel, with memantine's effect being significantly different from saline-treated controls even at the lowest dose tested. Once rats were familiarized with the testing environment, memantine, but not ketamine increased ambulatory activity at the high doses tested. Regardless of the context of the environment, the effects of ketamine on behavior in an

activity monitor were more affected by testing delay than those of memantine. The lowest dose of memantine or ketamine tended to increase spatial working memory as assessed in the spontaneous alternation task compared to controls at the shorter testing delay. However, the higher doses of either drug impaired performance in this task, which did not depend on the time of testing after drug administration. Furthermore, memantine, but not ketamine, elicited perseverative behavior; this effect was more pronounced when animals were tested 45 min after drug administration. Overall, our results indicate that the behavioral effects of memantine tended to be left-shifted and longer lasting compared to those of ketamine, consistent with the longer pharmacokinetics of memantine compared to that of ketamine.

4.2 INTRODUCTION

The clinical use of NMDA receptor inhibitors has been limited because they tend to induce symptoms similar to the disease of schizophrenia (Palmer, 2001; Lipton, 2004b). Ketamine, in particular, reliably reproduces the positive, negative, and cognitive symptoms of schizophrenia in healthy adults as well as exacerbating symptoms in already afflicted individuals (Cromhout, 2003). Although not widely used clinically, ketamine has been critical in furthering our understanding of the underlying pathology of schizophrenia. The NMDA receptor antagonist memantine, which inhibits NMDA receptor activity with similar affinity and kinetics as ketamine (Parsons et al., 1993; Mealing et al., 1999), lacks the psychotomimetic side-effects associated with many other NMDA antagonists and has been approved as a treatment for late-stages of moderate to severe Alzheimer's disease (Witt et al., 2004).

The behavioral effects of memantine versus ketamine in rats have been examined previously (Hetzler and Wautlet, 1985; Danysz et al., 1994; Barnes et al., 1996; Creeley et al., 2006; Koros et al., 2007); however, direct comparisons between these studies are difficult because of differences in rat strain, age, sex (Wilson et al., 2007), time of testing after drug administration, as well as route of drug administration. Subanaesthetic doses (up to 100 mg/kg) of ketamine administered to rats was reported to increase locomotor activity, decrease rearing, cause deficits in spatial working memory, induce stereotypic behavior, and decrease social behavior (Hetzler and Wautlet, 1985; Danysz et al., 1994; Koros et al., 2007). Effects similar to those of ketamine were observed in rats administered memantine, although the extent of the effects were less pronounced and required higher doses (Danysz et al., 1994; Koros et al., 2007). Moreover, contrary to the results mentioned above, data from Creeley et al. (2006) show that memantine produces severe locomotor deficits at neuroprotective doses (20 mg/kg, i.p.). Creeley et al. (2006) furthermore found that memantine administration at doses lower than those needed for neuroprotection (5 and 10 mg/kg, i.p.) causes locomotor and memory retention deficits.

A key aspect of memantine and ketamine that has not been investigated in previous studies is the difference in pharmacokinetics that exists between them. Whereas the distribution half-life in rat after injection of ketamine is ~1 min (Cohen et al., 1973; White et al., 1982; Annetta et al., 2005), for memantine it is ~1 h (Spanagel et al., 1994; Parsons et al., 1999). In previous studies, behavioral testing was conducted at only one time point after drug administration (Danysz et al., 1994; Koros et al., 2007). The differences in pharmacokinetic profile between memantine and ketamine should result in different cerebrospinal fluid (CSF) concentrations of the drugs at any one time after administration, which, in turn, might give rise to differences in the behavioral effects of the two drugs.

The focus of the present study was to test whether the differences in pharmacokinetics between memantine and ketamine are a primary source of the difference in behavioral effects elicited by the drugs. We tested this by assessing and directly comparing the effects in rat, of varying doses (0 - 40 mg/kg) of memantine and of ketamine on exploratory behavior in an activity monitor and on spatial working memory in the spontaneous alternation task, at either 15 min or 45 min after drug administration.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats weighing 250-275 g (Hilltop Labs, Scottdale, PA, USA) at the beginning of the study were used. Rats were single-housed and allowed 24-72 hr to acclimatize to the housing facility before the start of daily handling. The housing facility was maintained at a 12/12 hr light-dark cycle (lights on 0700) with a temperature of 22 ± 1 ºC and a humidity level of 45 ± 5 %. Animals were given free access to standard rat food and tap water.

4.3.2 Drugs and drug administration

Memantine hydrochloride (Sigma-Aldrich Co., St. Louis, USA; Fisher Scientific, Pittsburgh, USA) and ketamine hydrochloride (Sigma-Aldrich Co., St. Louis, USA) were dissolved in sterile physiological saline (0.9 % NaCl) and administered intraperitoneally (i.p.) at a volume of 2

ml/kg. Solutions were refrigerated and used within 3 weeks after preparation. Solutions were allowed to warm to room temperature before administration.

4.3.3 Experimental procedures

All handling and testing was performed in the Rodent Behavior Analysis Core facility of the University of Pittsburgh. Testing was conducted during the light phase of the light-dark cycle. Rats were handled in the housing facility for 5-7 min each for 3 d before the start of behavioral testing. At the end of the handling period rats were assigned to one of 10 drug conditions: memantine vehicle (0.9% NaCl saline); one of 4 doses of memantine (either 5, 10, 20, or 40 mg/kg); ketamine vehicle (0.9% NaCl saline); or one of 4 doses of ketamine (either 5, 10, 20, or 40 mg/kg). In addition, rats were assigned to one of two delays between drug administration and testing (either 15 min or 45 min). The experimenter was blind to the drug condition until completion of the study. Each animal was assigned to only one drug dose and one test delay time. All rats were tested in both behavioral paradigms (described below), and the order of testing was consistent across all animals (exploratory activity test followed by spontaneous alternation task). Rats were tested in each behavioral paradigm once and allowed 48 h to recover before the next testing paradigm. Rats were euthanized by CO_2 following completion of testing.

4.3.4 Exploratory activity test

Exploratory activity was monitored the day after completion of the 3 day handling period. Rats were injected with drug in a holding room separate from the housing and testing rooms and

placed back in their respective holding cages until testing. Animals were tested individually, and were naive to the testing room until the start of the 30 min testing session. Ambient illumination of the testing room was ~10 lux. Testing began when rats were placed in the center of a square-shaped activity monitor (42.5 cm x 42.5 cm x 30 cm; Med-Associates Inc., St. Albans, VT). The experimenter was not present in the room during testing. The animals' horizontal and vertical movements were monitored with the aid of infra-red light beams and detectors located along the sides of the walls of the test arena. Beam interruptions were analyzed offline with the aid of Med-Associates proprietary software. After testing, rats were returned to their respective home cages and returned to the housing facility, and the test arena was wiped clean with Quatracide before testing the next subject.

4.3.5 Spontaneous alternation task

Rats were injected in the holding room and returned to their holding cages until testing, as described above. Ambient illumination of the testing room was ~30 lux. The testing apparatus was a plus-shaped maze (arm dimensions: 47.5 cm x 9.8 cm x 20.3 cm) with a square center platform (9.8 cm x 9.8 cm) (Lafayette Instruments, Lafayette, IN). Animals were tested individually. Testing began when rats were placed in the center of the maze. Rats were allowed to explore freely the maze for 15 min. Arm entries (order and frequency) were monitored and analyzed with the use of an overhead digital camera and tracking software (Actimetrics/Coulbourn Instruments, Whitehall, PA). The experimenter was not present in the room during testing. After testing, rats were returned to their respective home cages and to the housing facility, and the maze was wiped clean with Quatracide before testing the next subject. An alternation was defined as an entry into four different arms during five consecutive arm

entries. Alternation scores were calculated by dividing the total number of alternations made by an animal by the total number of possible alternations (i.e., the total number of overlapping quintuplets). This value was multiplied by 100 to yield percent alternation. Perseveration was defined as an entry into the same arm twice during three consecutive arm entries. Perseveration scores were calculated by dividing the total number of perseverations made by an animal by the total number of possible perseverations (i.e., the total number of overlapping triplets). This value was multiplied by 100 to yield percent perseveration.

4.3.6 Statistical analysis

Data are presented as group means ± standard errors of the mean (SEM). To address the questions of interest to us, we evaluated dose effects for each drug and delay separately using one-way analyses of variance (ANOVA's) followed by *post-hoc* Dunnett tests to determine for which doses the effect differed significantly from saline. We evaluated differences between delays for each drug and dose separately using Student's t-tests for independent groups. Similarly, we evaluated differences between drugs for each dose and delay separately using Student's t-tests for independent groups. For all analyses, the significance level was set to $p \leq 0.05$.

4.4 RESULTS

4.4.1 Memantine effects on behavior in the exploratory activity test

Animal behavior was assessed by monitoring rats' performance in a 30 min exploratory activity test. The rats' behavioral profile changed markedly over the 30 min testing session (Figure 12A, B), especially the behavior of saline-treated animals. Therefore, we focused our attention on the first 5 min of testing (Figure 12C), when the context of the testing environment was novel, and the last 5 min of testing (Figure 12D), when rats were relatively familiar with the test environment.

Memantine administered (i.p. injection) either 15 min or 45 min before testing resulted in a significant dose-dependent decrease in ambulatory distance during the first 5 min of testing [15 min, $F_{(4,36)} = 20.11$, $p \leq 0.01$; 45 min, $F_{(4,36)} = 5.85$, $p < 0.01$]. *Post-hoc* tests revealed that in animals tested 15 min after memantine administration, every dose of memantine caused a significant decrease in ambulatory distance, whereas in animals tested 45 min after memantine administration, only doses of 10 mg/kg or higher caused a significant reduction in distance travelled during the first 5 min (p's < 0.01, Figure 12C). Although there was a trend for ambulatory distance to be reduced less when animals were tested 45 min after memantine administration, the effect of delay was not significant at any of the doses tested (all p's > 0.05).

During the last 5 min of the 30 min test, when animals had become familiar with the test environment, the ambulatory distance travelled by saline animals was markedly reduced, as expected (compare Figure 12D with 12C). In contrast, animals treated with higher doses of memantine became more active; although this effect was observed only in animals tested 15 min after memantine administration. Whereas the dose effect was significant for the 15 min groups

[$F_{(4,36)}$ = 8.89, $p < 0.01$], it failed to reach significance in animals tested 45 min after drug administration ($p > 0.05$). *Post-hoc* tests revealed that at the early test delay, animals injected with 40 mg/kg memantine travelled significantly farther than saline controls during the last 5 min of the test ($p < 0.01$, Figure 12D). None of the delay effects were significant (all p's > 0.05).

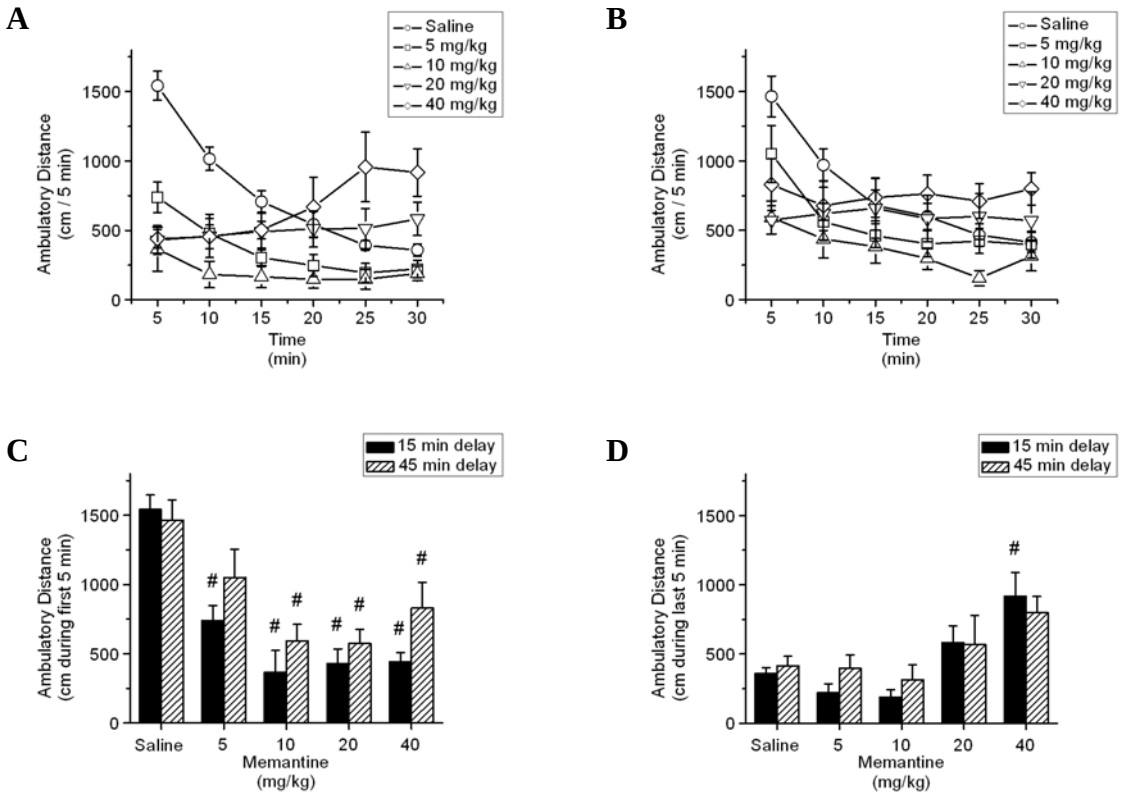

Figure 12. Effect of memantine on ambulatory distance in the exploratory activity test. A and B, Activity was averaged across 5 min intervals for the entire 30 min of testing. Memantine was administered either 15 min (A) or 45 min (B) before testing. The effect of time between memantine administration and testing on ambulatory distance in a novel and familiar

environment was assessed by examining the first (C) and last (D) 5 min of activity in the activity monitor, respectively. The results are displayed as means (cm/5 min) ± SEM of 9 (saline) or 8 (memantine doses) rats. # significantly ($p \leq 0.05$) different from respective saline controls (Dunnett test).

The loss in ambulatory distance we observed as a result of memantine administration in the first 5 min of activity testing cannot simply be attributed to a reduction in travel speed. Because, animals tested 15 min after memantine administration showed no significant effect of dose on travel speed. In animals tested 45 min after memantine administration, a significant dose-dependent decrease in travel speed was observed [$F_{(4,36)} = 4.83$, $p < 0.01$] because animals treated with the two highest doses tended to travel more slowly compared to saline-treated animals (20 mg/kg, $p < 0.01$; 40 mg/kg, $p < 0.05$, Table 3). Delay had no significant effect on travel speed (all p's >0.05). Memantine administration had no significant effect on travel speed during the last 5 min of open-field testing regardless of the delay before testing (p's > 0.05, Table 3). Taken together, these results suggest that the observed changes in distance travelled are not the result of a drug effect on travel speed. Rather, the reduction in ambulatory distance appears to be due to a reduction in the amount of time engaged in ambulatory activity.

Table 3. Effect of memantine on average velocity of ambulation in the exploratory activity test

	1st 5 min					Last 5 min				
	Saline	5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	Saline	5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg
15 min Delay	66.2 ± 4.0	45.2 ± 3.9	117.4 ± 26.1	57.1 ± 19.1	69.1 ± 17.1	73.9 ± 5.8	55.6 ± 15.4	47.1 ± 14.7	35.5 ± 6.2	60.1 ± 7.8
45 min Delay	65.3 ± 2.5	61.1 ± 3.8	51.3 ± 3.5	41.2 ± 5.6 #	49.1 ± 6.1 #	83.0 ± 10.3	60.9 ± 10.1	57.6 ± 10.9	45.7 ± 8.1	56.8 ± 8.4

The results are displayed as means (cm/sec) ± SEM of 9 (saline) or 8 (memantine doses) rats. # significantly ($p \leq 0.05$) different from respective saline controls (Dunnett test).

Much like we observed for ambulatory distance, the saline-treated animals showed a marked decrease in the number of times they reared over the course of the 30 min testing session (Figure 13A, B). Administration of memantine either 15 min or 45 min before testing resulted in a significant dose dependent decrease in rearings during the first 5 min of testing [15 min, $F_{(4,36)} = 42.98$, $p \leq 0.01$; 45 min, $F_{(4,36)} = 20.60$, $p < 0.01$]. *Post-hoc* tests revealed that at both delays every dose of memantine tested caused a significant decrease in the number of rearings (p's < 0.01, Figure 13C). Although there was a trend for rearings to be reduced less when animals were tested 45 min, compared to 15 min, after memantine administration, the effect of delay was only significant at the highest dose tested ($p < 0.05$).

Rearing behavior was also reduced for saline-treated animals during the last 5 min of testing (Figure 13D). A significant dose-dependent decrease in rearings was observed for both the 15 min and 45 min delay groups [15 min, $F_{(4,36)} = 21.22$, $p < 0.01$; 45 min, $F_{(4,36)} = 8.35$, $p < 0.01$]. *Post-hoc* tests revealed that at the early test delay, all memantine doses tested caused a significant decrease in the number of rearings compared to saline controls during the last 5 min of the test (all p's < 0.01, Figure 13D). When animals were tested 45 min after memantine administration, all but the lowest dose caused a significant decreased in the number of rearings (p's < 0.01). The drug effect on rearing during the last 5 min of the test was not sensitive to test delay (all p's > 0.05).

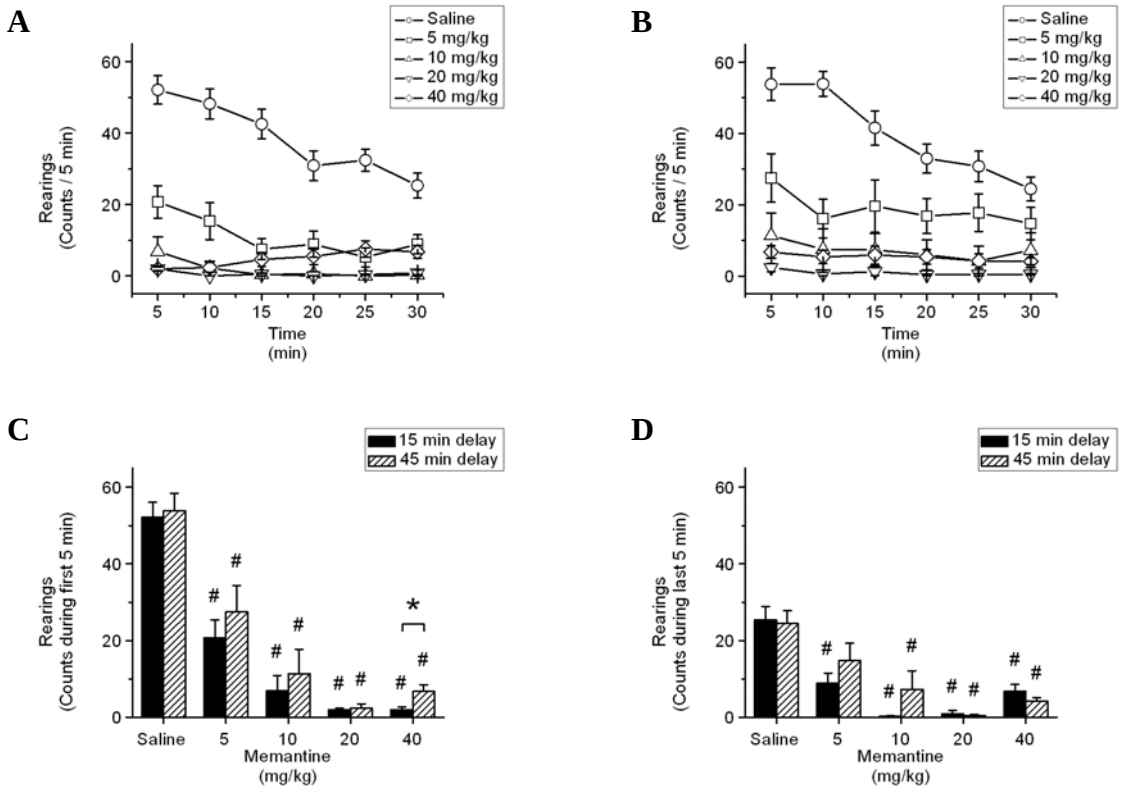

Figure 13. Effect of memantine on number of rearings in the exploratory activity test. A and B, Activity was averaged across 5 min intervals for the entire 30 min of testing. Memantine was administered either 15 min (A) or 45 min (B) before testing. The effect of time between memantine administration and testing on number of rearings in a novel and familiar environment was assessed by examining the first (C) and the last (D) 5 min of activity in the activity monitor, respectively. The results are displayed as means (counts/5 min) ± SEM of 9 (saline) or 8 (memantine doses) rats. # significantly ($p \leq 0.05$) different from respective saline controls (Dunnett test). * significantly ($p \leq 0.05$) different across delay (Student's t-test).

4.4.2 Memantine effect on behavior in the spontaneous alternation task

The effect of memantine on spatial working memory was assessed by monitoring rats' performance in a 15 min spontaneous alternation task. The spontaneous alternation task tests spatial working memory by taking advantage of a rats' natural tendency to explore its environment. As long as spatial working memory is not impaired, rats' will tend to explore the different arms of the plus-maze before re-entering an arm that has already been explored. Administration of memantine either 15 min or 45 min before testing resulted in a significant dose-dependent decrease in animals performances in the alternation task [15 min, $F_{(4,36)} = 5.97$, $p \leq 0.01$; 45 min, $F_{(4,36)} = 15.68$, $p < 0.01$]. *Post-hoc* tests revealed that in both delay groups, animals performances in the task were significantly reduced compared to saline controls when memantine was administered at either 20 or 40 mg/kg (all p's < 0.05, Figure 14A). No significant delay effect was observed at any of the doses tested (all p's > 0.05).

Upon analysis of the spontaneous alternation data, we noticed that some animals exhibited distinctly repetitive (perseverative) behavior, visiting the same two arms over and over. Therefore, we decided to analyze the data for this perseverative behavior (see Methods). Memantine caused a dose-dependent increase in this behavior whether administered 15 min [$F_{(4,36)} = 4.37$, $p < 0.01$] or 45 min [$F_{(4,36)} = 12.72$, $p < 0.01$] before testing. *Post-hoc* analyses showed that the 20 mg/kg group was significantly different from saline-treated animals at either testing delay, whereas 40 mg/kg group of memantine elicited a significant increase in perseverative behavior only at the longer delay between drug administration and testing (all p's < 0.01, Figure 14B). Accordingly, a delay effect was observed only at highest dose tested ($p < 0.05$).

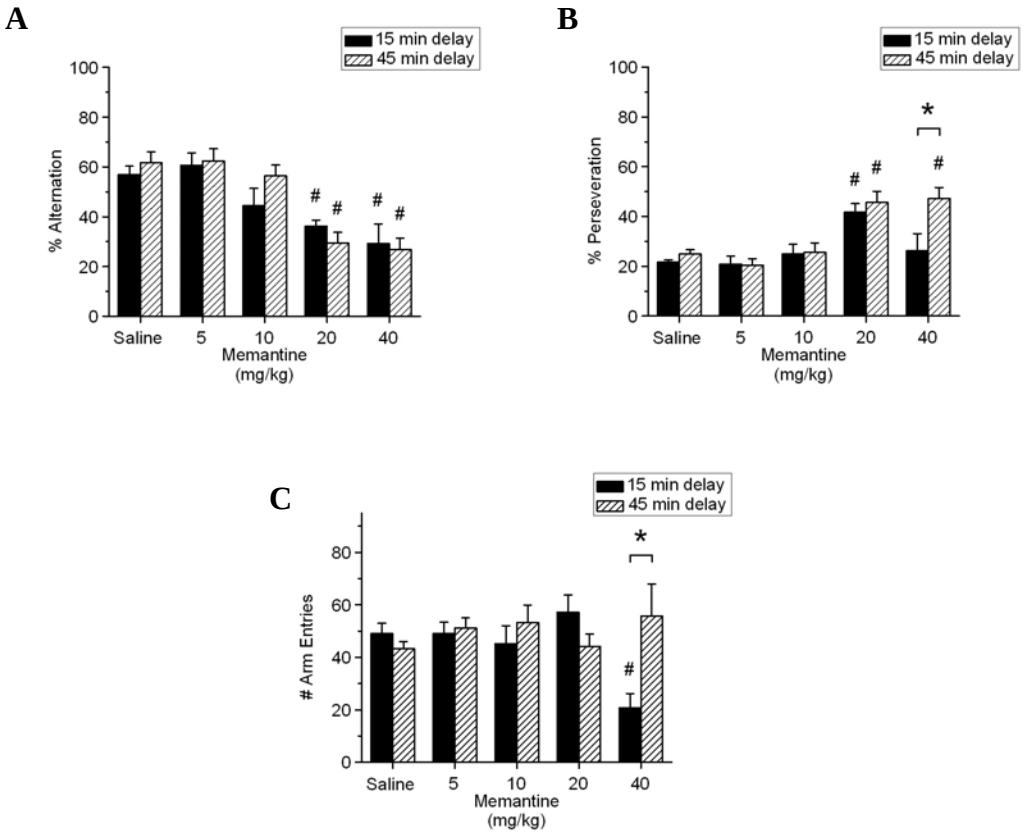

Figure 14. Effect of memantine on behavior in the spontaneous alternation task. A – C, The effect of memantine administered either 15 min or 45 min before testing on % alternation (A), % perseveration (B), and number of arm entries (C) in the plus-maze task. The results are displayed as means (%) ± SEM of 9 (saline) or 8 (memantine doses) rats. # significantly ($p \leq 0.05$) different from respective saline controls (Dunnett test). * significantly ($p \leq 0.05$) different across delay (Student's t-test).

The finding that perseverative behavior increased at the higher doses of memantine renders it unlikely that the decrease in alternation behavior at those doses was the result of a reduction in arm entries by animals that received memantine. Nevertheless, to examine a possible contribution of an overall decrease in maze exploration to the reduction in alternation, the total number of arm entries during the task was monitored and compared across conditions. The results revealed that the number of arm entries was reduced only by the highest dose of memantine tested and only at the 15 min delay between drug administration and testing [$F_{(4,36)} = 6.27$, $p < 0.01$]. A significant effect of delay on arm entries was observed for the 40 mg/kg dose of memantine ($p < 0.05$, Figure 14C). Thus, it cannot be ruled out that the decrease in alternation in the group that received 40 mg/kg of memantine and was tested 15 min after drug administration was not attributable, at least in part, to a decrease in arm entries.

4.4.3 Ketamine effects on behavior in the exploratory activity test

Similar to what we observed in animals treated with memantine (Figure 12A, B), the rats' behavioral profile changed markedly over the 30 min of testing in the activity monitor (Figure 15A, B). As previously, we focused our attention on the first 5 min, when the context was novel (Figure 15C), and the last 5 min of testing, when the test context was familiar (Figure 15D). Ketamine administered (i.p. injection) either 15 min or 45 min before testing resulted in a significant dose-dependent decrease in ambulatory distance during the first 5 min of testing [15 min, $F_{(4,36)} = 7.02$, $p \leq 0.01$; 45 min, $F_{(4,36)} = 8.81$, $p < 0.01$]. *Post-hoc* tests revealed that in animals tested 15 min after ketamine administration, all but the lowest dose tested caused a significant decrease in ambulatory distance (all p's < 0.05). In contrast, for animals tested 45 min after ketamine administration, only doses of 20 mg/kg or higher caused a significant

reduction in distance travelled during the first 5 min (p's < 0.05, Figure 15C). Ambulatory distance tended to be reduced less when animals were tested 45 min after ketamine administration; however, the effect of delay was only significant at the 5 mg/kg dose and the 20 mg/kg doses (p's < 0.05).

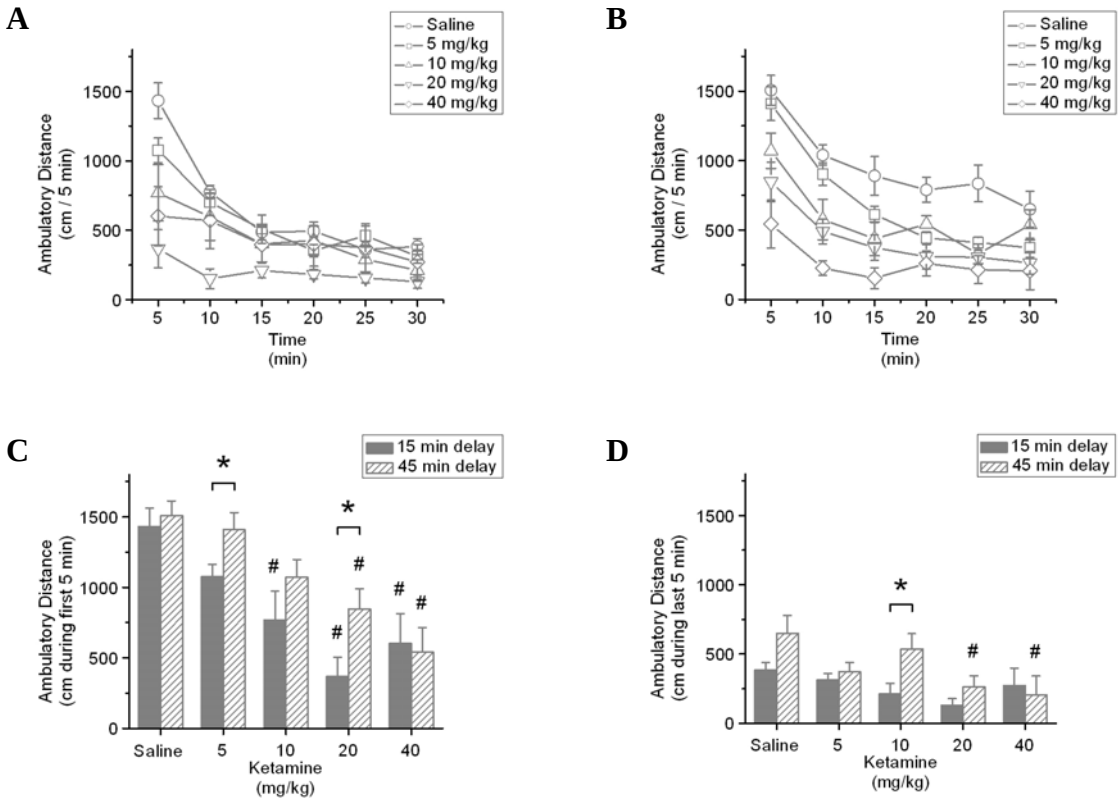

Figure 15. Effect of ketamine on ambulatory distance in the exploratory activity test. A and B, Activity was averaged across 5 min intervals over the entire 30 min of testing. Ketamine was administered either 15 min (A) or 45 min (B) before testing. The effect of time between ketamine administration and testing on ambulatory distance in a novel and a familiar

environment was assessed by examining the first (C) and last (D) 5 min of activity in the activity monitor, respectively. The results are displayed as means (cm/5 min) ± SEM of 9 (saline) or 8 (ketamine doses) rats. # significantly ($p \leq 0.05$) different from respective saline controls (Dunnett test). * significantly ($p \leq 0.05$) different across delay (Student's t-test).

As expected, ambulatory distance was reduced for saline-treated animals during the last 5 min of the 30 min test, when animals are familiarized with the test environment (Figure 15D). In contrast to memantine, ketamine had no effect on ambulatory distance traveled during the last 5 min in animals treated 15 min before testing ($p > 0.05$) and caused a significant dose-dependent decrease in animals tested 45 min after drug administration [$F_{(4,36)} = 2.98$, $p < 0.05$]. The results of *post-hoc* tests revealed that animals that received 20 mg/kg or 40 mg/kg of ketamine travelled significantly less than saline controls when tested 45 min after ketamine administration ($p \leq 0.05$, Figure 15D). Delay had a significant affect on ambulatory behavior during the last 5 min only in the groups that received 10 mg/kg of ketamine, with animals tested at the longer delay travelling further than animals tested at the short delay ($p < 0.05$).

Similar to our observations with memantine, the decrease in ambulatory distance as a result of ketamine administration during the first 5 min in the activity monitor cannot be attributed to a reduction in travel speed. Regardless of the delay between drug administration and the beginning of testing, no significant effect of dose or delay on ambulation speed were observed (p's > 0.05, Table 4). The decrease in ambulatory distance we observed as a result of ketamine administration during the last 5 min in the activity monitor also cannot simply be attributed to reduced travel speed. Although animals tested 15 min after ketamine administration

did show a significant dose-dependent effect on average velocity during the last 5 min [$F_{(4,36)}$ = 4.22, $p < .01$]. However, only the 20 mg/kg dose group was significantly reduced from saline-treated controls ($p < 0.05$, Table 4), a group that showed no significant effect on ambulatory distance. No significant dose effects were observed for the 45 min delay groups ($p > 0.05$). No significance of testing delay was measured at any dose (p's > 0.05).

Measurements of the number of rearings over the 30 min test session showed that rearings markedly changed over the testing session (Figure 16A, B). Much like memantine (Figure 13A, B), the administration of ketamine was associated with a large reduction in rearing behavior regardless of whether animals were tested 15 min or 45 min after drug administration [15 min, $F_{(4,36)} = 20.52$, $p \leq 0.01$; 45 min, $F_{(4,36)} = 18.51$, $p < 0.01$]. *Post-hoc* tests revealed that at both delays, each but the lowest dose of ketamine tested here caused a significant decrease in rearing behavior (p's < 0.01, Figure 16C). Rearings tended to be reduced less when animals were tested 45 min after ketamine administration; however, the effect of delay was significant only for the 5 mg/kg and 20 mg/kg doses ($p < 0.05$).

Ketamine also caused a significant reduction in rearing behavior during the last 5 min of the 30 min test in animals tested 15 min as well as animals tested 45 min after drug administration [15 min, $F_{(4,36)} = 15.21$, $p < 0.01$; 45 min, $F_{(4,36)} = 7.06$, $p < 0.01$]. The results of *post-hoc* tests revealed that at the early test delay, all but the lowest ketamine dose tested significantly decreased number of rearings compared to saline controls during the last 5 min of the test (all p's < 0.05, Figure 16D). In contrast, when tested 45 min after ketamine administration, only the highest dose tested significantly decreased number of rearings (p's < 0.01). Delay effects were significant only for the 20 mg/kg dose ($p < 0.05$).

Table 4. Effect of ketamine on average velocity of ambulation in the exploratory activity test

	1st 5 min					Last 5 min				
	Saline	5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	Saline	5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg
15 min Delay	66.0 ± 3.6	55.8 ± 1.9	64.2 ± 10.4	40.1 ± 13.9	48.1 ± 14.7	62.7 ± 2.9	69.3 ± 5.5	45.9 ± 14.6	24.6 ± 10.1 #	38.7 ± 7.0
45 min Delay	61.8 ± 4.6	60.0 ± 2.4	57.3 ± 3.6	62.0 ± 4.6	50.9 ± 13.2	59.8 ± 5.4	51.0 ± 9.4	62.3 ± 9.6	42.6 ± 11.2	34.4 ± 18.8

The results are displayed as means (cm/sec) ± SEM of 9 (saline) or 8 (ketamine doses) rats. # significantly ($p \leq 0.05$) different from respective saline controls (Dunnett test).

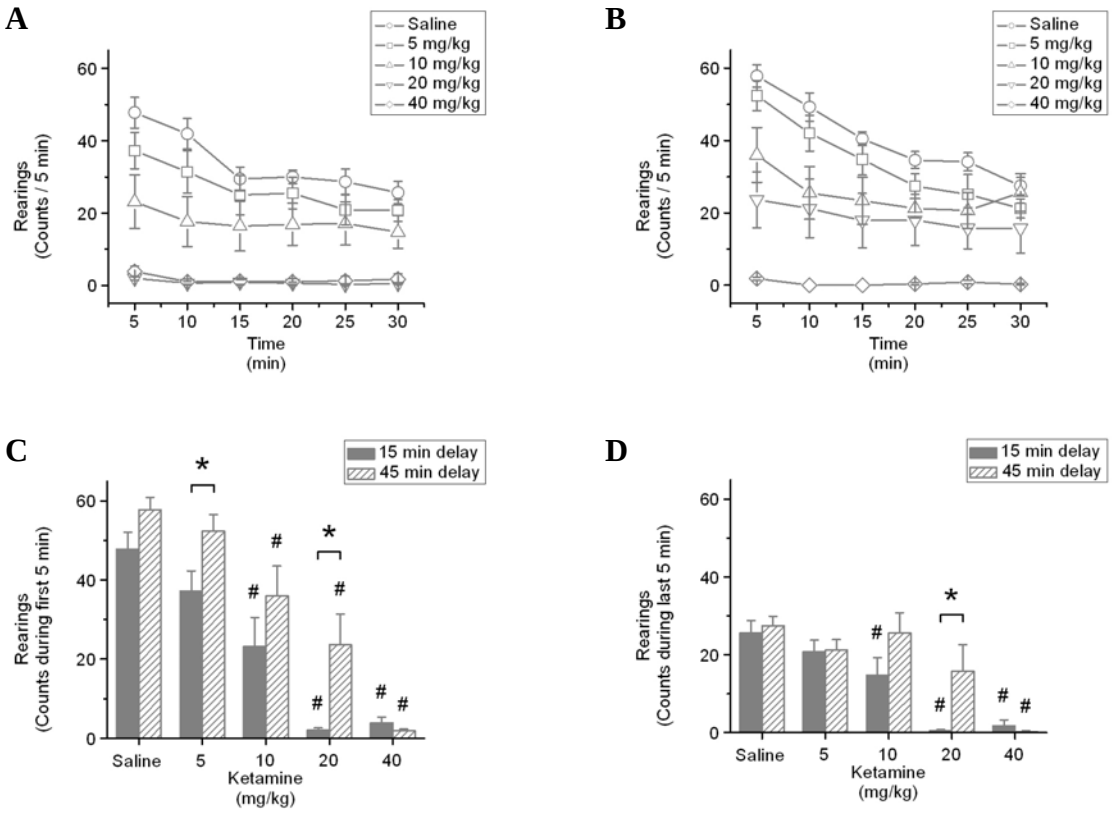

Figure 16. Effect of ketamine on number of rearings in the exploratory activity test. A and B, Activity was averaged across 5 min intervals over the entire 30 min of testing. Ketamine was administered either 15 min (A) or 45 min (B) before testing. The effect of time between ketamine administration and testing on rearings in a novel or familiar environment was assessed by examining the first (C) and last (D) 5 min of activity in the activity monitor, respectively. The results are displayed as means (counts/5 min) ± SEM of 9 (saline) or 8 (ketamine doses) rats. # significantly ($p \leq 0.05$) different from respective saline controls (Dunnett test). * significantly ($p \leq 0.05$) different across delay (Student's t-test).

4.4.4 Ketamine effects on behavior in the spontaneous alternation task

Ketamine effects on spatial working memory were assessed by monitoring rats' performance in a spontaneous alternation task. Ketamine administration either 15 min or 45 min before testing on the plus-maze resulted in a significant dose-dependent decrease in alternation behavior [15 min, $F_{(4,36)} = 4.02$, $p \leq 0.01$; 45 min, $F_{(4,36)} = 4.06$, $p < 0.01$]. *Post-hoc* tests showed that ketamine produced a significant reduction in alternation behavior from saline control levels only at the highest dose tested but this reduction was observed at both test delays ($p < 0.05$, Figure 17A). No significant delay effect was observed at any of the doses tested (all p's > 0.05).

Administration of ketamine either 15 min or 45 min before testing did not significantly affect perseverative behavior (p's > 0.05, Figure 17B). The total number of arm entries also was not affected when animals were tested 15 min after ketamine administration ($p > 0.05$). However, when animals were tested 45 min after drug administration, ketamine caused a significant decrease [$F_{(4,36)} = 8.85$, $p < 0.01$], which was restricted to the two highest doses tested (20 mg/kg and 40 mg/kg, p's < 0.01, Figure 17C). We observed no significant delay effects on the total number of arm entries as a result of the administration of ketamine (all p's > 0.05). Our findings that the highest dose of ketamine caused a reduction in alternation behavior at both test delays but a reduction in arm entries only at the long delay further support our claim that the drug-associated decrease in alternation behavior cannot readily be explained in terms of a drug-induced reduction in arm entries.

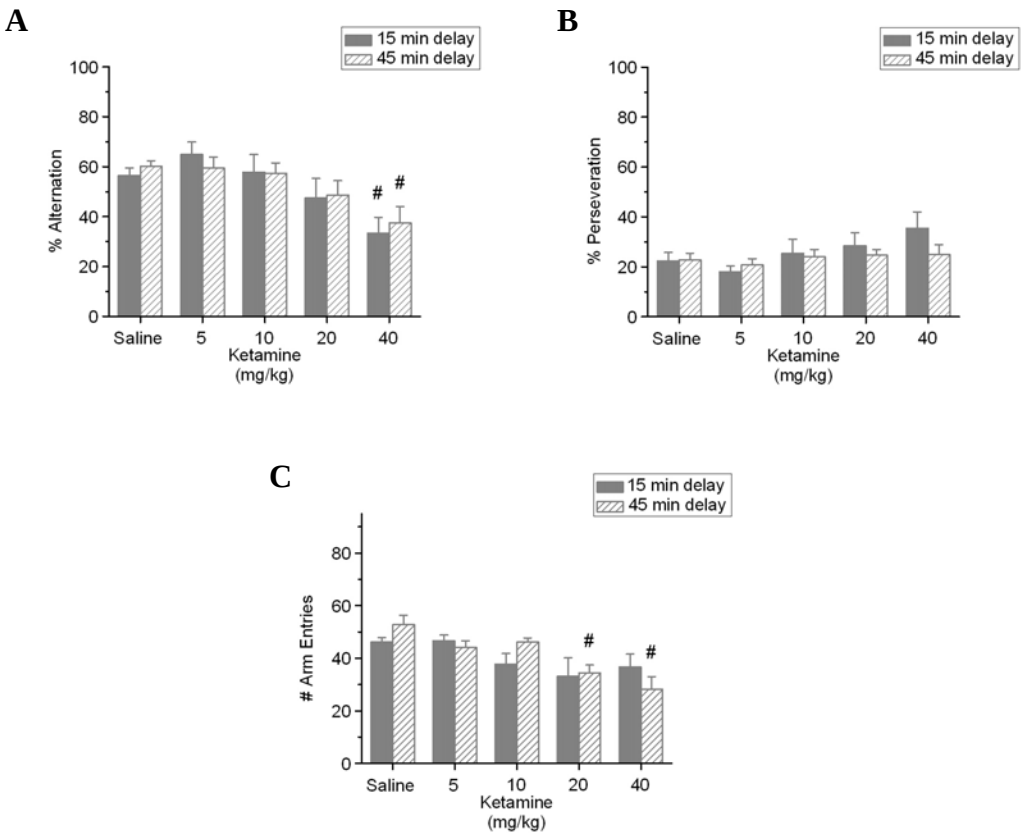

Figure 17. Effect of ketamine on behavior in the spontaneous alternation task. A – C, The effect of ketamine administered either15 min or 45 min before testing on % alternation (A), % perseveration (B), and number of arm entries (C) in the plus-maze task. The results are displayed as means (%) ± SEM of 9 (saline) or 8 (ketamine doses) rats. # significantly ($p \leq 0.05$) different from respective saline controls (Dunnett test).

4.4.5 Comparison of the effects of memantine and ketamine on behavior in the exploratory activity test

The effects of memantine and ketamine on the tested behaviors were directly compared by subtracting each individual animal's score from the average of the saline controls. Drug-induced effects were analyzed separately for each test delay after drug administration. Furthermore, as previously, attention was focused on the first 5 min and last 5 min of the test, when the context of the testing environment was novel and familiar, respectively.

These comparisons revealed that, generally, the suppressive effect of memantine and ketamine on ambulation during the first 5 min of the exploratory activity test were more pronounced after administration of memantine than of ketamine. This trend was statistically significant at the lowest dose when the drugs were administered 15 min before testing ($p < 0.05$, Figure 18A), and at the second lowest dose (10 mg/kg) when they were administered 45 min before testing ($p < 0.05$, Figure 18B). The high doses (20 and 40 mg/kg) of both drugs decreased ambulatory distance to the same extant, possibly as a result of a floor effect (achieved at the 10 mg/kg dose for memantine). Overall the data suggest that the effects of memantine on ambulatory distance in a novel environment are left-shifted compared to those of ketamine.

Memantine and ketamine had more diverse effects on ambulatory distance during the last 5 min of exploratory activity testing. The higher doses of memantine increased ambulatory distance, especially at the shorter testing delay, whereas no such effect was observed with ketamine. Accordingly, the effect of memantine and ketamine on ambulatory behavior during the last 5 min was found to differ significantly from one another at the two highest doses (20 and 40 mg/kg) when administered 15 min before testing (p's < 0.01, Figure 18C) and at the highest dose (40 mg/kg) when administered 45 min before testing ($p < 0.01$, Figure 18D).

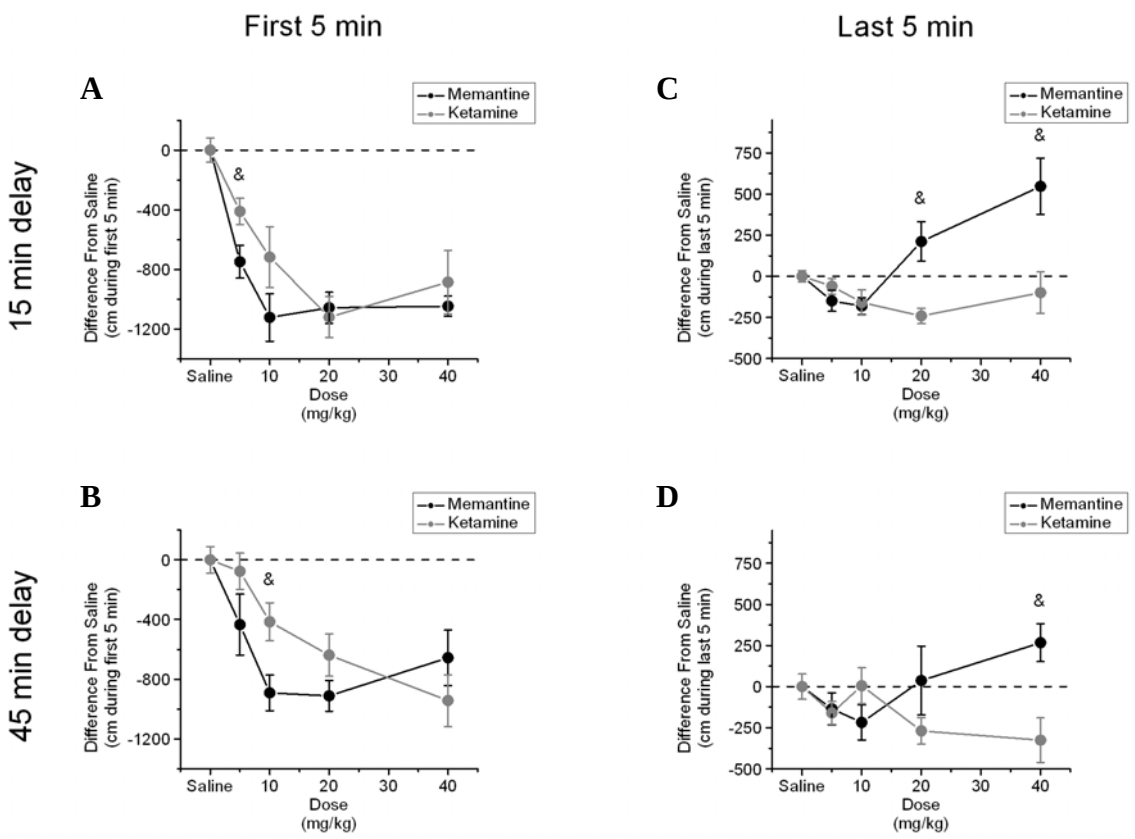

Figure 18. Comparison of memantine's and ketamine's effect on ambulatory distance in the exploratory activity test. The effect of memantine or ketamine administered either 15 min (A, C) or 45 min (B, D) before testing on ambulatory distance was analyzed during the first 5 min of the test , when the environment was novel (A, B), and the last 5 min of the test when the environment was familiar (C, D). The results are displayed as means (cm/5 min) ± SEM of 18 (saline) or 8 (drug doses) rats. & significant ($p \leq 0.05$) difference from corresponding drug dose (Student's t-test).

Similar to the pattern of effect on ambulation, memantine revealed to cause a greater reduction in rearing behavior than did ketamine, and this effect was observed at both testing delays as well as during the first and the last 5 min of testing. During the first 5 min of testing this trend was significant only at the lowest dose tested (5 mg/kg) when the drugs were administered 15 min before testing ($p < 0.05$, Figure 19A), possibly due to a maximum effect at all higher doses. When administered 45 min before testing, memantine caused a significantly greater reduction in rearing compared to ketamine at all doses tested, except that at the highest dose tested, a small increase in rearing from the suppressed level by memantine treated animals resulted in a reversal in relation between the drug effects that revealed also to be statistically significant (all p's < 0.05, Figure 19B). The greater suppressive effect of memantine, compared to ketamine, on rearing also was evident during the last 5 min of the exploratory activity test. However, at the highest dose tested here, the relationship between the drugs again was reversed. Thus, when administered 15 min before testing, memantine caused a greater reduction in rearing during the last 5 min at 5 mg/kg and 10 mg/kg, but less of a reduction at 40 mg/kg (all p's $\leq$ 0.05, Figure 19C). When administered 45 min before testing, memantine caused a greater reduction in rearing at 10 mg/kg and 20 mg/kg, whereas the relationship was reversed at 40 mg/kg (all p's < 0.05, Figure 19D). Like we observed during the first 5 min of testing, memantine's effects on rearings during the last 5 min appeared to be left-shifted compared to the effects of ketamine, and a trend that was more obvious at the longer test delay because ketamine's effect appeared to begin wearing off, especially at the lower doses.

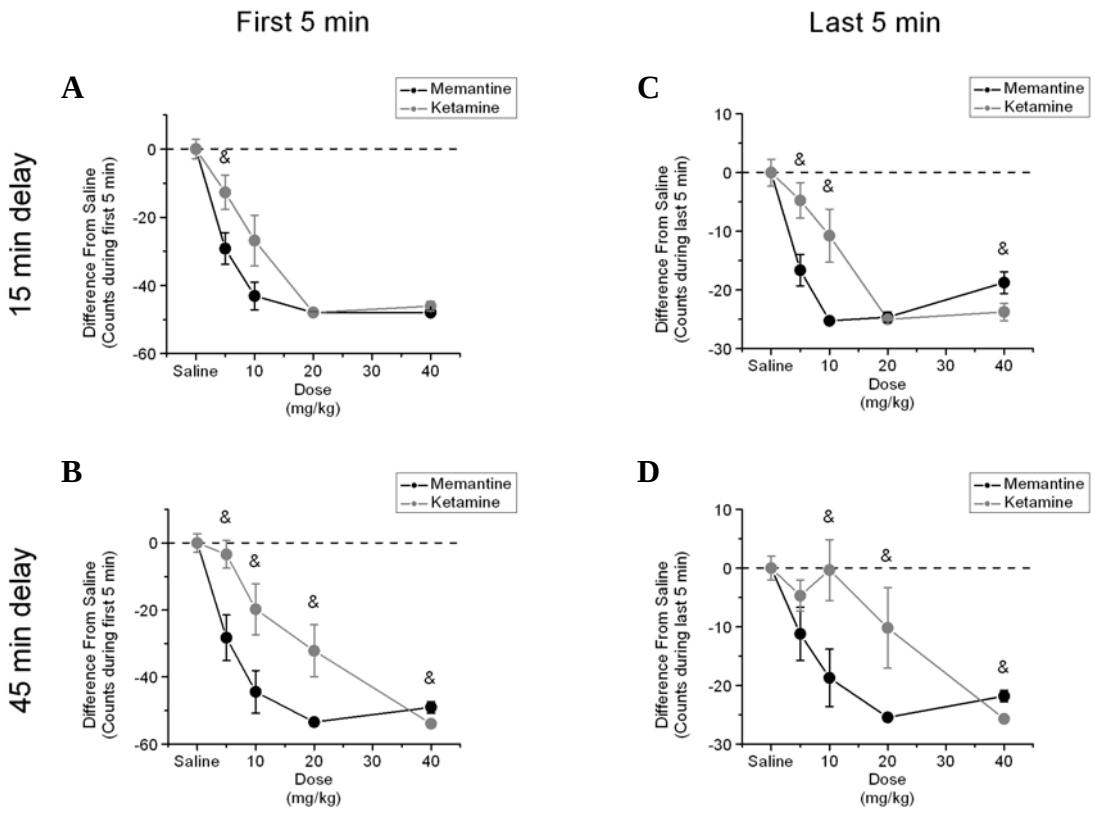

Figure 19. Comparison of memantine's and ketamine's effects on number of rearings in the exploratory activity test. The effect of memantine or ketamine administered either 15 min (A, C) or 45 min (B, D) before testing on number of rearings was analyzed for the first 5 min of the test, when the environment was novel (A, B) and for the last 5 min of the test when the environment was familiar (C, D). The results are displayed as means (counts/5 min) ± SEM of 18 (saline) or 8 (drug doses) rats. & significant ($p \leq 0.05$) difference from corresponding drug dose (Student's t-test).

4.4.6 Comparison of the effects of memantine and ketamine on behavior in the spontaneous alternation task

Overall, a similar effect on alternation behavior was observed following administration of either memantine or ketamine. Only the high doses of either drug decreased rats' performance in spatial alternation regardless of the time between drug administration and testing (Figure 20A, B). Although memantine tended to be more effective than ketamine at reducing performance in the alternation task at both delays, significant differences between drugs was achieved only for the 20 mg/kg dose administered 45 min before testing ($p < 0.05$, Figure 20B). In Contrast, the effects of memantine or ketamine on perseverative behavior in this task were markedly different. Animals tested 15 min after administration of 20 mg/kg of memantine showed a significant increase in the occurrence of perseverative behavior compared to ketamine ($p \leq 0.05$, Figure 20C). When testing began 45 min after dug administration, a significantly greater increase in perseverative behavior was observed in memantine-treated animals' compared to ketamine-treated animals' at both the 20 mg/kg and the 40 mg/kg dose ($p < 0.01$, Figure 20D).

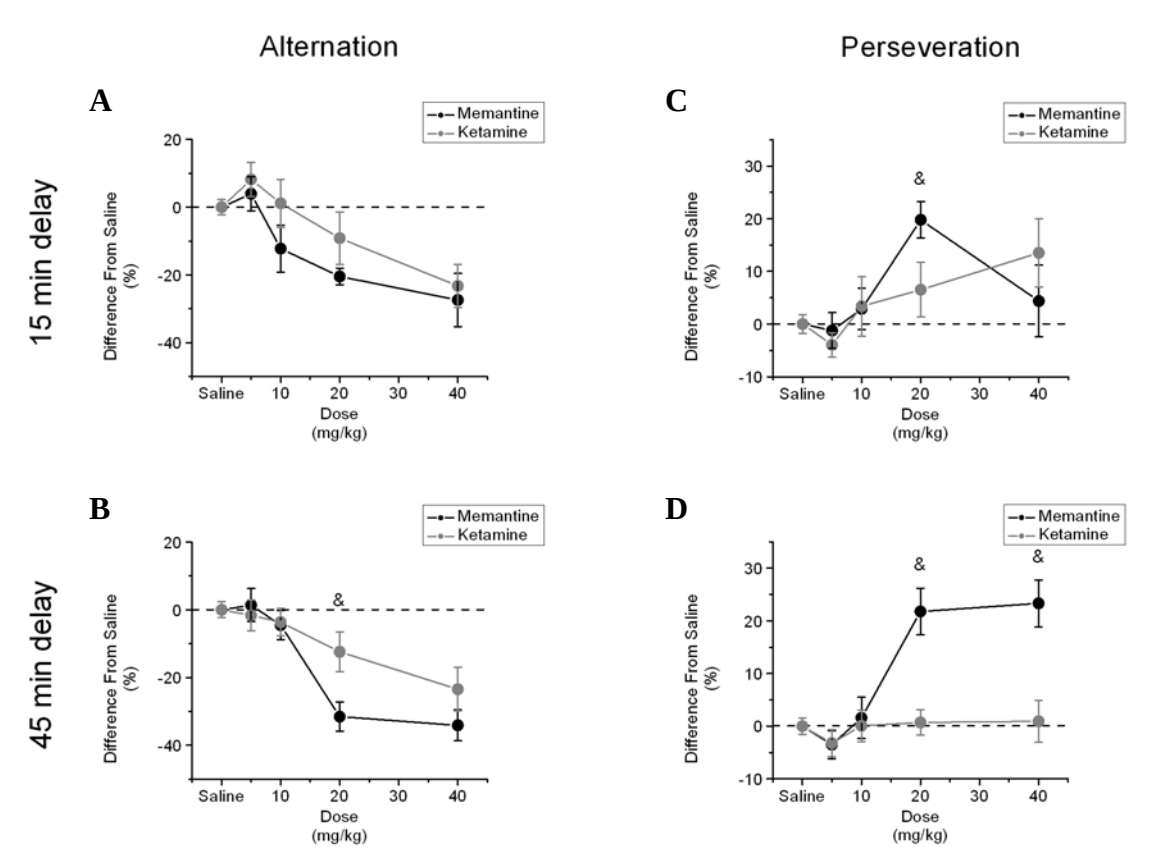

Figure 20. Comparison of memantine's and ketamine's effect on behavior in the spontaneous alternation task. Depicted is the effect of either memantine or ketamine administered either 15 min (A, C) or 45 min (B, D) before testing on % alternation (A, B) and % perseveration (C, D) in the plus-maze. The results are displayed as means (%) ± SEM of 18 (saline) or 8 (drug doses) rats. & significantly ($p \leq 0.05$) different from corresponding drug dose (Student's t-test).

4.4.7 Comparison of the effects of memantine and ketamine on general behavior in the exploratory activity test

The time rats spent in four distinct types of activity (resting, ambulating, non-travel movement (limb or head movements made while the animals' body was stationary), and rearing) during the 30 min in the activity monitor were measured. These values were transformed into percent time spent in each activity over the entire 30 min of testing. These data reveal that the rats spent a majority of the test session resting regardless of the drug administered or the time of testing after administration (Figure 21). The percent time rats rested was dramatically increased by administration of memantine or ketamine even at the lowest dose (5 mg/kg) tested. The increase in resting time resulting from administration of memantine or ketamine was closely associated with a decrease in time spent rearing. The percent time spent ambulating as well as in non-travel movement was not greatly affected by administration of memantine or ketamine at the doses tested.

Comparing the effects of memantine and ketamine on time spent in the activities measured at either delay demonstrates that, at the lower doses (5 and 10 mg/kg) tested, memantine's effects appear left-shifted compared to those of ketamine. This left-shift appears more prominent when rats were tested 45 min (Figure 21B, D) rather than 15 min (Figure 21A, C) after drug administration, seemingly due to a greater reduction of the drug effects among ketamine-treated animals. Another distinct difference between the drugs in their effect on activity in the activity monitor was that the higher doses (20 and 40 mg/kg) of memantine caused an increase in relative time spent ambulating and engaging in non-travel movement, whereas this trend did not develop among ketamine-treated animals.

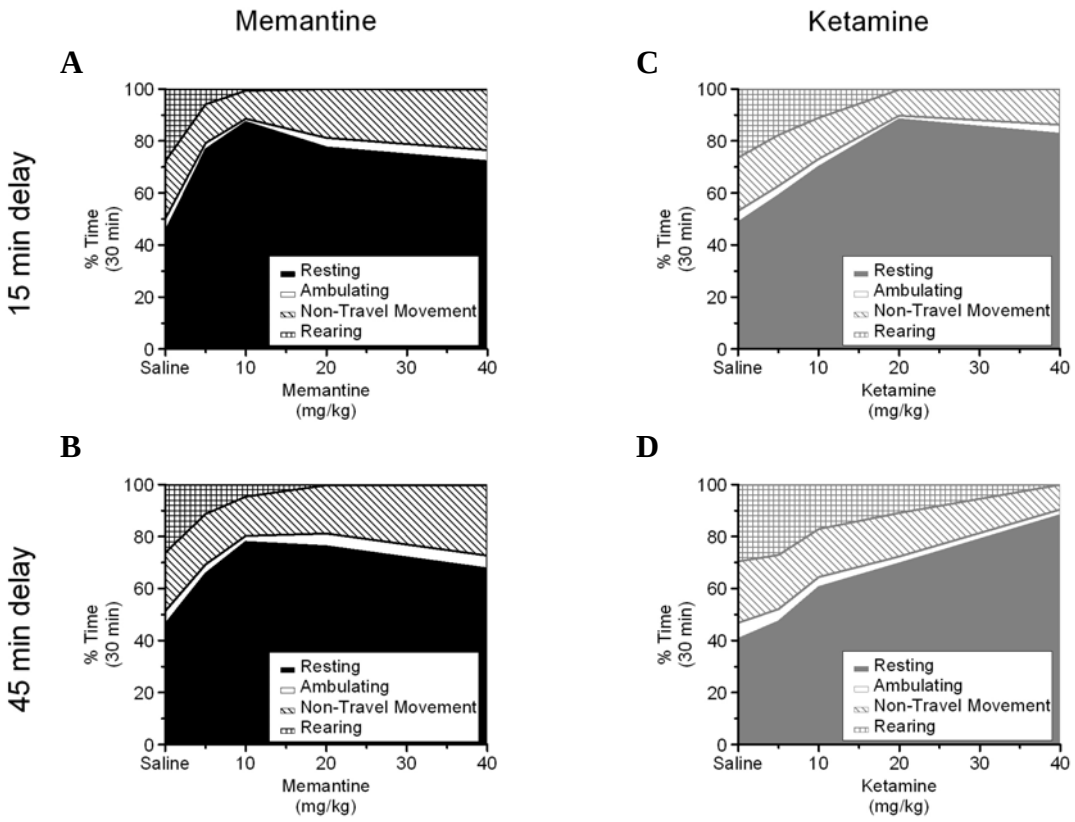

Figure 21. Comparison of memantine's and ketamine's effect on general behavior in the exploratory activity test. Depicted is the effect of either memantine (A, B) or ketamine (C, D) administered either 15 min (A, C) or 45 min (B, D) before testing on the % time spent in various activities (resting, ambulating, non-travel movement, and rearing) in the activity monitor. The results are displayed as the percent of total time spent in each activity for 9 (saline) or 8 (drug dose) rats.

4.5 DISCUSSION

We administered varying doses of either memantine or ketamine via i.p. injection either 15 min or 45 min before examining behavior in the exploratory activity test and, on a separate occasion, behavior in the spontaneous alternation task. We used these two time delays between drug administration and testing to assess the effect of previously reported pharmacokinetic differences between these two drugs (Cohen et al., 1973; White et al., 1982; Spanagel et al., 1994; Parsons et al., 1999; Cromhout, 2003; Annetta et al., 2005) on behavior. We found that administration of memantine induced similar, yet more pronounced and longer lasting behavioral changes than did ketamine. In addition, memantine, at the higher doses, evoked some behavioral trends not observed in animals treated with similarly high doses of ketamine.

4.5.1 Memantine and ketamine effects on behavior in the exploratory activity test

At the doses tested, and at both testing delays, memantine (Figure 12C, 18A, B) and ketamine (Figure 15C, 18A, B) dose-dependently reduced ambulatory behavior during the first 5 min of exploratory activity testing compared to saline controls. The effects of memantine appeared to be less affected by the delay before testing, whereas ketamine's effects tended to wane over the longer test delay. However, in contrast to our observations of a reduction in ambulatory activity, previous studies (Hetzler and Wautlet, 1985; Danysz et al., 1994; Koros et al., 2007) found that these two NMDA receptor antagonists tended to increase locomotor behavior. A possible explanation for the apparent discrepancy, is that the test environment was novel to the rats in our study, whereas rats had been habituated to the test environment in the previous studies (Hetzler and Wautlet, 1985; Danysz et al., 1994; Koros et al., 2007). Thus, it is possible that under

conditions of a novel environment, both memantine and ketamine have a suppressive effect on ambulatory behavior. In contrast, under conditions of a familiar environment, the NMDA receptor antagonists tend to increase ambulatory behavior. Consistent with this possibility, we found, similar to the previous reports (Danysz et al., 1994; Koros et al., 2007), that during the last 5 min of the exploratory activity test, when the ambulatory behavior of saline-treated animals was markedly reduced because of habituation to the test environment, animals treated with the highest dose of memantine we tested, 40 mg/kg, exhibited a significant increase in ambulatory distance compared to saline controls (Figure 12D, 18C, D). Ketamine, on the other hand, never elicited an increase in ambulatory distance during the last 5 min of exploratory activity testing at the doses we tested (Figure 15D, 18C, D). These results appear to conflict with the previous reports that ketamine induces increased locomotor activity (Hetzler and Wautlet, 1985; Danysz et al., 1994); however, it should be noted that in these previous studies, the doses of ketamine that elicited an increase in locomotor activity were higher (> 50 mg/kg) than those tested here. One study (Koros et al., 2007) did report a significant increase in locomotor activity in rats administered a dose of ketamine (s.c.) as low as 8 mg/kg. The divergent effects elicited by memantine and ketamine on ambulatory activity in a familiar environment suggest there may be a fundamental difference in the actions of these two drugs when administered at higher doses.

Memantine and ketamine, dose-dependently decreased rearing behavior in the exploratory activity test, regardless of the degree of novelty of the test environment or the testing delay. Previous studies also found that these drugs decrease rearing (Danysz et al., 1994; Koros et al., 2007). Our results demonstrate that, although similar, memantine's effect on rearing behavior is more pronounced and longer lasting than is ketamine's effect (Figure 19).

The left-shift of memantine's effects on general activity in the activity monitor compared to ketamine's effects at the low-to-moderate doses (5 and 10 mg/kg) tested here and the wearing off of ketamine's effects at the longer delay are clearly demonstrated in Figure 21. This figure also demonstrates the divergent effects of the higher drug doses (20 and 40 mg/kg) tested.

4.5.2 Memantine and ketamine effects in the spontaneous alternation task

Animals were tested in the spontaneous alternation task to asses the effects of memantine and ketamine on cognitive functioning, specifically on spatial working memory. A previous study by Jackson et al. (2004) demonstrated that administration of a low dose of another, higher affinity, NMDA receptor antagonist, MK-801 (0.01 mg/kg, i.p.), increased performance in this task; however, higher doses ($\geq$ 0.05 mg/kg) significantly impaired performance compared to saline-treated controls. Here, we did not observe a significant increase in alternation behavior upon administration of either memantine or ketamine, although a slight trend toward enhanced alternation emerged at the lowest dose tested (5 mg/kg). It is possible that a lower dose of memantine or ketamine could have resulted in a statistically significant enhancement of performance in the alternation task. Higher doses of memantine and ketamine tested in our study tended to reduce spontaneous alternation, consistent with what Jackson et al. (2004) observed with MK-801. Although memantine's effects in this task tended to be more prominent than ketamine's, this trend generally was not statistically significant (Figure 20A, B).

The effects of memantine and ketamine on perseverative behavior measured with the plus-maze reveals a fundamental difference between the actions of these two drugs. At the doses tested, ketamine had no affect on perseverative behavior (Figure 17B), whereas memantine caused a dramatic increase in perseverative behavior at the higher dosestested (Figure 14B).

4.5.3 Examination of the behavioral effects elicited by memantine and ketamine

The main site of action of both memantine and ketamine is thought to be NMDA receptors (Bormann, 1989; Kornhuber et al., 1989; Yamamura et al., 1990; Witt et al., 2004). It is surprising that memantine has more profound behavioral effects than ketamine, given that electrophysiological data suggest that ketamine has a higher affinity for NMDA receptors (Parsons et al., 1993; Parsons et al., 1995; Parsons et al., 1996; Mealing et al., 1999). Although ketamine is a more effective inhibitor of NMDA receptor-mediated responses than memantine, the pharmacokinetics of memantine were found to be much slower than those of ketamine (White et al., 1982; Spanagel et al., 1994; Cromhout, 2003). The slower pharmacokinetics of memantine make it likely that a given dose of memantine will accumulate in the cerebrospinal fluid (CSF) at a higher concentration than does the same dose of ketamine. The higher drug concentration in the CSF will allow memantine to more effectively inhibit NMDA receptor-mediated responses, explaining the left-shift of the behavioral effects we observed when comparing the effects of the lower doses (5 and 10 mg/kg) of the two drugs. The slower pharmacokinetics of memantine could also explain why memantine's effects, when assessed 45 min after drug administration, tended to be comparable to those observed 15 min after drug administration. In the case of ketamine, on the other hand, significant differences between the two test delays emerged more frequently, likely due to more rapid wearing off of the drug's effects.

The divergent effects of the higher doses of memantine and ketamine tested here suggest one of two possibilities; 1) the effects of memantine and ketamine on some behaviors are best represented by a U-shaped curve, and/or 2) memantine and ketamine differentially affect other neurotransmitter systems at the higher doses tested. Some of the data gathered in this study

(such as locomotor activity) suggest a U-shaped dose-response curve for memantine. It is possible that a similar trend would be observed with ketamine as well had we tested yet higher doses. Consistent with this possibility, previous studies (Hetzler and Wautlet, 1985; Danysz et al., 1994) measuring ketamine's effect on locomotor activity noted an increase in activity at doses of 50 mg/kg and higher. Regardless of the shape of the dose-response curves, it is highly likely that effects on other neurotransmitter systems, in addition to an effect on NMDA receptors, occur at the higher doses tested here. Both memantine and ketamine affect other neurotransmitter systems at concentrations comparable to those measured for NMDA receptor inhibition (Parsons et al., 1995; Blanpied et al., 1997; Danysz et al., 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Mealing et al., 1999; Coates and Flood, 2001; Rammes et al., 2001; Kashiwagi et al., 2002; Maskell et al., 2003; Aracava et al., 2005; Seeman et al., 2005; Chen and Lipton, 2006; Seeman et al., 2008).

Compared to each other, both memantine and ketamine demonstrate a similar affinity for NMDA receptors regardless of subunit composition (Yamakura et al., 1993; Bresink et al., 1996; Parsons et al., 1999). Therefore, it is unlikely that the different effects of the high doses of these drugs reported here are a result of differential inhibition at NMDA receptor subtypes. Another receptor that has been hypothesized to play a role in psychotic behavior is the functional high-affinity state of the D_2 dopamine receptor (D_2^{High}) (Seeman et al., 2006). However, action at the D_2^{High} receptor is also an unlikely explanation for the difference in behavioral effects of high doses of memantine versus ketamine, because both drugs are high-affinity agonists at D_2^{High} receptors (Seeman et al., 2005; Seeman et al., 2008). The affinity of these drugs for D_2^{High} receptors is comparable to that for NMDA receptors; therefore, any effect on behavior as a result of the drugs' actions on D_2^{High} receptors should not be limited to just one of the two drugs.

It is unclear how effects at other neurotransmitter systems may have contributed to the divergent behavioral responses we observed at the highest doses of the drugs tested in this study. In summary, our results suggest that pharmacokinetics can have an important effect on the type and extent of behavioral changes elicited by a given dose of drug. Investigators need to be aware of pharmacokinetic differences between drugs when comparing behavioral effects measured at a single time point after administration.

5.0 GENERAL DISCUSSION

Proper NMDA receptor functioning is crucial for the accurate transmission of synaptic signals within the vertebrate CNS. Alterations in NMDA receptor-mediated neurotransmission via pharmacological agents, injury, or disease can lead to debilitating effects on behavior and cognition. Attempts at pharmacologically restoring normal NMDA receptor function due to insult or disease have been met with varied success. The research presented in this dissertation was performed in an attempt to understand the success and failure of two mechanistically similar NMDA receptor antagonists, memantine and ketamine, as therapeutic agents.

5.1 INTEGRATION OF BEHAVIORAL AND ELECTROPHYSIOLOGICAL FINDINGS

The behavioral and cognitive effects of memantine and ketamine were directly assessed in adult male Sprague-Dawley rats. Overall, our results suggest that memantine and ketamine, at low-to-moderate doses, induce similar behavioral and cognitive effects in rat; however, the effects of memantine tended to be more pronounced at a given dose. The behavioral and cognitive changes measured with low-to-moderate doses of memantine appeared to be less affected by the time of testing after drug administration than ketamine. At higher doses, however, memantine and ketamine had divergent effects, which may reflect actions of these drugs at CNS targets other

than NMDA receptors. In order to integrate our behavioral findings with our electrophysiological findings we will ignore the actions of these drugs at alternate targets until later.

We measured memantine's and ketamine's ability to inhibit activity of recombinant NMDA receptors. We report that memantine is slightly less effective than ketamine at inhibiting NMDA receptor activity in the absence of Mg^{2+}_o. We also report that NR2 subunit composition did not greatly affect inhibition by either drug.

The reported overlap of the binding site of memantine and ketamine with that for Mg^{2+}_o (Sobolevsky et al., 1998; Kashiwagi et al., 2002) suggests that competition is likely to occur between these drugs and endogenous Mg^{2+}_o. To better understand the actions of memantine and ketamine on NMDA receptor activity in the CNS we measured the ability of these drugs to inhibit NMDA receptor-mediated responses near resting potential in the presence of a physiologically relevant concentration (1 mM) of Mg^{2+}_o. The presence of 1 mM Mg^{2+}_o decreased the ability of memantine and ketamine to inhibit NMDA receptors composed of the NR1 and any NR2 subunit. However, the decrease in inhibitory ability was more prominent for NR1/2A or NR1/2B receptors than for NR1/2C or NR1/2D receptors. In the presence of physiological Mg^{2+}_o, ketamine was still slightly more effective than memantine at inhibiting NMDA receptor activity at all NR2 subunits except at receptors composed of the NR1/2D subunit. Our results suggest that physiological Mg^{2+}_o imparts NMDA receptor subunit specificity to the inhibitory effects of memantine and ketamine; however, the shift in IC_{50} values were similar for the two drugs. Because of the prevalence of the NR2A and NR2B subunit in the cortex, a majority of research has examined memantine's and ketamine's interaction with NMDA receptors composed these NR2 subunits. An important finding of our results is that

NR2C and/or NR2D containing NMDA receptors may be more involved in physiology and disease than previously appreciated. These findings alone are unlikely, however, to explain the varying behavioral and cognitive effects that we observed at high doses, since we observed relatively small differences in the subunit specificity of memantine compared to ketamine.

Another aspect of NMDA receptor inhibition by memantine and ketamine that we examined was the degree to which these drugs become trapped within the channels of receptors that have closed and unbound agonist. It has been reported (Blanpied et al., 1997; Chen and Lipton, 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998; Mealing et al., 1999), and our results agree, that ketamine is more fully trapped within closed NMDA receptor channels than memantine. We found evidence supporting the hypothesis (Figure 6) that the differences in trapping of memantine and ketamine is the result of memantine interacting with NR1/2A receptors at two binding sites, a deep trapping site (shared by both drugs) and a superficial non-trapping site. The partial trapping of memantine should allow some recovery from inhibition between successive synaptic signals. Whereas the more complete trapping of ketamine should result in no recovery from inhibition between synaptic events, leading to greater inhibition of NMDA receptor mediated activity. It remains unclear whether NR2 subunit composition affects the degree of trapping of memantine or ketamine, especially at NR1/2C or NR1/2D receptors.

At first glance our behavioral and electrophysiological findings appear contradictory. How can it be that, at the same dose, greater behavioral and cognitive effects were elicited by memantine which is generally less effective at inhibiting NMDA receptor activity (their main site of action) than ketamine? A likely explanation lies in the pharmacokinetic differences between memantine and ketamine. In humans the elimination half-life of ketamine (~2.5 h) is

much faster than for memantine (60-80 h) (White et al., 1982; Spanagel et al., 1994; Parsons et al., 1999; Cromhout, 2003; Annetta et al., 2005; Johnson and Kotermanski, 2006). If this difference in elimination half-life is similar in rat, then a higher concentration of memantine than ketamine should build up in the brain after administration of a given dose. Thus, a greater concentration of memantine will be present in the CNS to inhibit NMDA receptor activity than ketamine. Our observation that the behavioral effects induced by memantine are less affected by the time of testing after administration than ketamine is in agreement with this line of thinking.

The more profound behavioral and cognitive effects of memantine, compared to ketamine, at lower doses may be attributable to its longer pharmacokinetic profile. However, the longer elimination half-life of memantine allows for a therapeutic dose to be practically administered as a once-a-day oral tablet. The faster elimination half-life of ketamine would likely require multiple daily dosings to achieve a comparable steady state concentration of drug in the CSF. Thus, the slower pharmacokinetics of memantine compared to ketamine is likely to contribute to its greater therapeutic benefit. It should be noted that long pharmacokinetics is not always desirable in a clinical situation. While useful for prolonged or chronic treatments, the longer pharmacokinetics of a drug makes quick changes in plasma levels more difficult. The fast pharmacokinetics of ketamine is likely to contribute to its usefulness as an anesthetic, where tight control of drug plasma levels is a necessity.

5.2 POSSIBLE CONSEQUENCES OF NR2C AND/OR NR2D RECEPTOR INHIBITION

As demonstrated in this document, the NMDA receptor antagonists memantine and ketamine are more effective at inhibiting NR1/2C or NR1/2D receptors, than NR1/2A or NR1/2B receptors, in the presence of physiological Mg^{2+}_{o}. This finding suggests the need for a greater understanding of the role NR2C and NR2D containing receptors play in the functioning of the CNS.

In adults, the NR2C subunit of NMDA receptors exhibits higher levels of expression, compared to other brain regions only in the cerebellar granule cells (Monyer et al., 1992; Ishii et al., 1993; Monyer et al., 1994; Wenzel et al., 1996; Cull-Candy et al., 2001). It is unclear how NMDA receptor antagonism of NR2C containing receptors in the cerebellum may contribute to the behavioral and cognitive consequences of memantine or ketamine administration. Antagonism at NR2C containing receptors and disruption of cerebellar activity would likely result in ataxia, which appeared to occur at the higher doses of memantine and ketamine tested but was not measured. The effects of memantine and ketamine on cerebellar functioning could be measured by testing rat performance on a rotorod. However, our interest is more on the behavioral effects of these drugs which are likely mediated by forebrain, where NR2C expression is low (Monyer et al., 1992; Ishii et al., 1993; Monyer et al., 1994; Wenzel et al., 1996; Cull-Candy et al., 2001). Therefore, this discussion will focus more on the effects of antagonism at NR2D receptors.

Although levels are lower than for NR2A or NR2B containing receptors, NR2D containing receptors are present extrasynaptically in the adult cortex and on inhibitory interneurons in the hippocampus (Monyer et al., 1994; Akbarian et al., 1996; Standaert et al., 1996; Wenzel et al., 1996; Dunah et al., 1998; Fleidervish et al., 1998; Munoz et al., 1999;

Thompson et al., 2002; Lozovaya et al., 2004; Binshtok et al., 2006; Karavanova et al., 2007). In situations where relatively low CSF concentrations of memantine or ketamine favor inhibition of NR2D containing receptors over NR2A or NR2B, excitatory neurotransmission may be enhanced. Extrasynaptic NR2D containing receptors, which are less inhibited by Mg^{2+}_o than those containing NR2A or NR2B, should be more active in pathologically compromised cortical neurons. This is likely to increase the baseline level of activity of those neurons and a resultant decrease in the ability to detect physiologically relevant signals (Parsons et al., 2007). Preferential inhibition of extrasynaptic NMDA receptors that contain the NR2D subunit could help to decrease the heightened level of background noise hypothesized to occur in Alzheimer's disease (Parsons et al., 2007). Furthermore, preferential inhibition of NR2D containing receptors expressed on interneurons would decrease interneuron firing and as a result may enhance physiologically relevant signals (Krystal et al., 2003). This proposed mechanism of preventing pathological activity while preserving, or possibly enhancing, physiological stimuli may explain the beneficial cognitive effects of relatively low CSF concentrations memantine. Memantine's slightly better ability to inhibit NR2D containing receptors than ketamine may contribute to the greater therapeutic utility of memantine. Related to this idea, our behavioral results demonstrated a slight but insignificant enhancement of rats' performance in a spatial working memory task after acute administration of a dose (5 mg/kg, i.p., Figure 20A) considered to result in a therapeutically relevant brain concentration of memantine (Parsons et al., 2007). However, we also observed this increase in spatial working memory with a corresponding dose of ketamine. It is possible that an even lower dose may have enhanced animals' performance in the task even further. Although this is consistent with the beneficial actions of memantine in humans (Parsons et al., 1999; Kilpatrick and Tilbrook, 2002; Witt et al., 2004), it is not

consistent with the actions of ketamine, which does not appear to enhance cognitive functioning in humans (Krystal et al., 1999a; Krystal et al., 2003).

When the brain concentration of memantine or ketamine reaches a level sufficient to achieve significant inhibition at all NMDA receptor subtypes, NMDA receptor-mediated transmission may become sufficiently disrupted to resemble the hypofunction associated with schizophrenia (Aghajanian and Marek, 2000; Coyle et al., 2003), thereby inducing schizophrenic-like symptoms (Newcomer et al., 1999; Aghajanian and Marek, 2000; Coyle et al., 2003; Northoff et al., 2005). NMDA receptor antagonism has been shown to preferentially decrease activity of inhibitory neurons, leading to disinhibition (Greene, 2001). Possible explanations for the increased sensitivity of interneurons to NMDA receptor inhibition, compared to excitatory pyramidal neurons, include differential NMDA receptor subtype expression (Monyer et al., 1994; Akbarian et al., 1996; Standaert et al., 1996; Wenzel et al., 1996; Dunah et al., 1998; Fleidervish et al., 1998; Munoz et al., 1999; Greene, 2001; Thompson et al., 2002; Lozovaya et al., 2004; Binshtok et al., 2006; Karavanova et al., 2007), and greater excitatory drive to interneurons that could lead to less Mg^{2+}_{o} block of NMDA receptors (Povysheva et al., 2006). A result of NMDA receptor antagonist-induced disinhibition is an increase in glutamate release (Moghaddam and Jackson, 2003). The higher baseline level of glutamate (Krystal et al., 1999a; Krystal et al., 2003) that results from disinhibition of interneuron activity would be likely to make it more difficult for physiological stimuli to be detected. The decreased ability to detect physiologically relevant stimuli, as a result of increased glutamate release in the cortex, may contribute to the observed decrease (hypofrontality) in performance of schizophrenic patients (Weinberger and Berman, 1996), as well as in individuals administered ketamine (Northoff et al., 2005), on tasks mediated by the prefrontal cortex.

5.3 POSSIBLE BENEFITS OF PARTIAL VERSUS FULL TRAPPING

A possible contributor to the greater tolerability of low doses of memantine compared to ketamine in humans may be the difference in degree of trapping by NMDA receptors. The partial trapping of memantine should allow some (~17 %) synaptic receptors to recover from block between successive releases of glutamate from presynaptic terminals. This should allow for greater detection of subsequent physiologically relevant signals, while still effectively decreasing the baseline level of NMDA receptor activity at the postsynaptic neuron, thereby presumably preserving (although likely decreasing) physiological activity. The mechanism of partial trapping effectively results in greater inhibition of chronically active NMDA receptors than those activated by brief pulses of agonists. The partial trapping of memantine suggests that it can never completely block NMDA receptor response to successive presynaptic releases of glutamate. It is unclear if partial trapping of memantine occurs at NR2C or NR2D containing receptors, or how partial trapping by NMDA receptors composed of these subunits may contribute to the tolerability of memantine.

The more complete trapping of ketamine will likely not allow for recovery of receptors from the blocked state between successive presynaptic releases of glutamate. This should result in a greater depression of NMDA receptor mediated transmission including a more profound disturbance of physiological activity, which may underlie ketamine's lower tolerability in humans compared to memantine. It should be noted that at high concentrations of memantine and ketamine, NMDA receptor activity should be so disrupted that any beneficial effect of partial trapping could be lost. Examining differences in trapping of memantine and ketamine at NR2C or NR2D containing receptors, where we show they are likely to exert their action in the CNS, may help further our understanding of the therapeutic and behavioral effects of these drugs.

5.4 POSSIBLE EFFECTS ON OTHER NEUROTRANSMITTER SYSTEMS

We found that physiological Mg^{2+}_o decreased the ability of memantine or ketamine to inhibit activity at their main site of action, NMDA receptors, near resting membrane potentials. Thus, although NMDA receptor mediated transmission is still likely to be greatly affected by memantine or ketamine, the actions of these drugs are not as NMDA receptor-specific as previously imagined.

Memantine has been reported to inhibit serotonin ($5\text{-}HT_3$) (Danysz et al., 1997; Rammes et al., 2001; Chen and Lipton, 2006) and nicotinic acetylcholine (nACh) ($\alpha 7$) receptors (Maskell et al., 2003; Aracava et al., 2005; Chen and Lipton, 2006), as well as having agonist actions at dopamine receptors (D_2^{High}) (Seeman et al., 2008). These actions of memantine have been reported to occur at concentrations comparable those that inhibit NMDA receptors. Ketamine demonstrates antagonistic effects at $\alpha 7$ nACh receptors (Coates and Flood, 2001) and agonist effects at D_2^{High} dopamine receptors (Seeman et al., 2005) at concentrations comparable to those reported for memantine.

Of particular importance to schizophrenia are the actions of memantine or ketamine on the dopaminergic system. The high-affinity state of the D_2 dopamine receptor (D_2^{High}) has been hypothesized to play a role in psychosis (Seeman et al., 2006). Both memantine and ketamine demonstrate, with similar affinity, agonist actions at the D_2^{High} receptor (Seeman et al., 2005; Seeman et al., 2008). Both drugs have also been shown to increase dopamine release in the cortex of rats (Verma and Moghaddam, 1996; Moghaddam et al., 1997; Hesselink et al., 1999; Lorrain et al., 2003). While these actions on the dopaminergic system may contribute to the

similar behavioral and cognitive effects of these two drugs we measured in rats, they are unlikely to explain their different tolerability in humans.

The serotonergic system is differentially affected by memantine and ketamine. Ketamine is a much weaker antagonist at 5-HT_3 serotonin receptors than memantine (Rammes et al., 2001; Kos et al., 2006). It is possible that the differential actions of memantine and ketamine at 5-HT_3 receptors may affect the behavioral and cognitive effects of these drugs. 5-HT_3 receptors are present in many brain regions thought to be involved in schizophrenia, including the cortex (Hagan et al., 1993). 5-HT_3 antagonists can decrease hyperactivity of dopaminergic neurons (Faerber et al., 2007), an effect that should be beneficial in alleviating the symptoms of schizophrenia (Seeman, 2002; Stone et al., 2007). Thus any psychotomimetic effects of memantine that result from its ability to increase dopamine release may be partially counteracted by inhibition of 5-HT_3 receptors. Ketamine's similar ability to release dopamine, coupled with its weaker inhibition of 5-HT_3 receptors, could result in stronger psychotomimetic effects. However, it should be mentioned that 5-HT_3 receptor antagonists were not beneficial to schizophrenic individuals in clinical trials (Faerber et al., 2007). Further research needs to address the actions of memantine and ketamine on neurotransmitter systems other than the glutamatergic system, and how effects on these systems may contribute to the varying tolerability reported for low doses of these drugs in humans. Contributions of other neurotransmitter systems to the behavioral and cognitive deficits elicited by high doses of memantine and ketamine are even more likely, and should be examined as well.

5.5 CONCLUDING COMMENTS

The findings reported in this document demonstrate the difficulties inherent in attempting to correlate basic research results into a comprehensive account of the actions exerted by a given drug in humans and other animals. The simplification of a system to examine specific effects of a drug is an important, and as of yet, only way to gather a detailed understanding of a drug's effects. However, the physiological environment in which drugs are exerting their actions ultimately cannot be ignored and may profoundly alter their effects at a specific target. Species related differences between animal models used for research and humans complicates the application of basic research findings to understanding of drug actions on human CNS functioning. Species differences in drug distribution and metabolism will greatly affect the actions elicited by a given drug on the CNS functioning of the animal, as well as the resultant behavioral and cognitive effects. For instance, rodent metabolism is much faster than human metabolism, resulting in faster elimination of a given drug dose from the rodent CNS than from the human CNS (Parsons et al., 2007). Furthermore, the human cortex is much more highly developed than the rodent cortex (Robbins, 1998). Differences in neurotransmitter receptor distribution and connections among the different neurotransmitter systems that are likely to exist across species makes comparisons of drug effects even more difficult. Addressing these complications will likely further our ability to use findings gathered through diverse research techniques to increase our understanding of the human consequences of a drug's action.

APPENDIX

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